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Sfrp1 promotes neuroinflammation through the modulation of ADAM10 proteolytic activity

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INDEX

PRESENTACIÓN

Una creciente evidencia indica la importancia de la respuesta inmune en la regulación y el mantenimiento de la homeostasis del tejido neural. La pérdida de esta homeostasis puede estar entre las posibles causas que contribuyen a la aparición o el desarrollo de desórdenes neurológicos. Los procesos inflamatorios en el Sistema Nervioso Central (SNC) están mediados por astrocitos y microglia, protegiéndolo frente a cualquier tipo de daño en el intento de reparar el tejido. Sin embargo, cuando estos procesos inflamatorios son exacerbados o dilatados en el tiempo inducen neurotoxicidad. La neuroinflamación crónica ha sido implicada como uno de los detonantes de las diversas enfermedades neurodegenerativas y autoinmunes que atacan al SNC.

Trabajo previo de nuestro laboratorio ha demostrado que Sfrp1 contribuye a la progresión de la enfermedad de Alzheimer (EA), promoviendo la generación de péptidos Aβ. Durante este estudio observamos que la inactivación genética de *Sfrp1* va asociada a unos niveles reducidos de inflamación. Esto unido a varios estudios que relacionan altos niveles de expresión de Sfrp1 con enfermedades preiféricas asociadas a una inflamación crónica, nos llevó a hipotetizar que Sfrp1 podría estar directamente implicado en la regulación de la neuroinflamación.

Esta cuestión ha sido abordada a lo largo de esta tesis, aportando evidencias que la sustentan. Hemos demostrado que la expresión de Sfrp1 se ve incrementada en migroglia y astrocitos bajo diversas condiciones experimentales, usando modelos animales de neuroinflamación, de EA o Encefalomielitis Autoinmune Experimental. Por el contrario, la inactivación genética de *Sfrp1* reduce severamente la activación glial, provocando una mejora de los signos patológicos de los modelos. En cambio, la sobreexpresión de Sfrp1 es suficiente para inducir una respuesta inflamatoria, mientras que estudios preliminares indicamos que la neutralización de la actividad de Sfrp1 mejora la patología de la EA. En relación al posible mecanismo de acción, Sfrp1 parece promover la neuroinflamación al regular el procesamiento de varios sustratos de ADAM10 implicados en la activación de las células microgliales: TREM2, CD200 y CX3CL1.

Por tanto, proponemos una implicación directa de Sfrp1 en la modulación de la actividad microglial durante la neuroinflamación, lo que sugiere que podría representar una nueva diana terapéutica para atenuar los exacerbados procesos neuroinflamatorios presentes en numerosas enfermedades neurodegenerativas.

ABSTRACT

Growing evidence suggests the importance of immune response regulation for the maintenance of neural tissue homeostasis. Disruption of this homeostasis might be one of the causes contributing to the onset and development of neurological disorders. Inflammatory responses in the Central Nervous System (CNS) are mediated by astrocytes and microglial cells, which help to protect from pathogen invasion and respond to any kind of injury, in the attempt to repair the tissue. However, exacerbated inflammatory responses lead to pathogenic neurotoxicity and chronic neuroinflammation. The latter has been recognized as one of the drivers of diverse neurodegenerative and autoimmune diseases of the CNS.

Previous work from our laboratory has demonstrated that Sfrp1 contributes to AD progression by inducing Aβ peptide generation. In the course of this study, we also observed that genetic inactivation of *Sfrp1* was associated with particularly low levels of neuroinflammation. Because an increased Sfrp1 expression has been reported in several peripheral diseases associated with chronic inflammation, we hypothesised that Sfrp1 could directly contribute to the regulation of neuroinflammation.

In this thesis, we have addressed this issue, providing evidence that support this hypothesis. Indeed, we show that Sfrp1 expression is upregulated in activated microglial cells and reactive astrocytes under diverse experimental pro-inflammatory conditions, including experimentally induced neuroinflammation, in mouse models for Alzheimer's Disease (AD) and in Experimental Autoimmune Encephalomyelitis. On the contrary, genetic inactivation of *Sfrp1* strongly reduces glial cells activation, ameliorating the pathological traits of the diseases. Sfrp1 overexpression is sufficient to induce an inflammatory response, activating glial cells and promoting the infiltration of immune cells, whereas preliminary studies indicate that antibody-mediated neutralization of Sfrp1 activity ameliorates AD pathological traits. From a mechanistic point of view, Sfrp1 seems to promote neuroinflammation by regulating ADAM10-mediated shedding of TREM2, CD200 and CX3CL1, proteins implicated in the activation of microglial cells.

We thus propose that Sfrp1 is directly involved in modulating microglial activation during brain inflammation. We also suggest that Sfrp1 may represent a new therapeutic target to attenuate the exacerbated neuroinflammation present in numerous neurodegenerative diseases.

ABBREVIATIONS

AD	Alzheimer's Disease
ADAM	A Disintregrin And Metalloprotease
ApoE	Apolipoprotein E
APP	Amyloid Precursor Protein
Arg1	Arginse 1
Aβ	Amyloid Beta
BACE	β-site APP Cleaving Enzyme
BBB	Blood Brain Barrier
BSA	Bovine Serum Albumin
Ca	Calcium
cc	Corpus Callosum
CD	Cluster of Differentiation
CNS	Central Nervous System
CO_2	Carbon Dioxide
CR	Complement Receptor
CRD	Cysteine Rich Domain
CSF	Colony-Stimulating Factor
DAMP	Damage-Associated Molecular Pattern
DAP12	DNAX Activation Protein 12
DMEM	Dulbecco’s modified Eagle medium
DNA	DeoxyriboNucleic Acid
E	Embryonic day
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	EthyleneDiamineTetraacetic Acid
EGTA	EthyleneGlycolTetraacetic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
Fc	Fragment crystallisable
FCS	Fetal Calf Serum
Fz	Frizzled
GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
GFAP	Glial Fibrillary Acid Protein
GFP	Green Fluorescent Protein
GSK	Glycogen Synthase Kinase
H_2O_2	Hydrogen Peroxide
HBSS	Hank’s Balanced Salt Solution
HCl	HydroChloric acid
HEK	Human Embryonic Kidney cells
Iba1	Ionized calcium Binding Adaptor 1
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
IRES	Internal Ribosome Entry Site
ITAM	Immune-receptor Tyrosine-based Activator Motive
ITIM	Immune-receptor Tyrosine-based Inhibition Motive
L	Ligand

LPS	Lipopolysaccharide
LV	Lateral Ventricle
Mac1	Macrophage-1 antigen (AKA CD11b)
MBP	Myelin Basic Protein
Mg	Magnesium
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	messenger RNA
MS	Multiple Sclerosis
NaCl	Sodium Chloride
NFκB	Nuclear Factor Kappa-chain-enhancer of activated B cells
NICD	Notch IntraCellular Domain
NSC	Neural Stem Cell
NTR	Netrin Related Domain
P	Postnatal day
P-	Phosphorylated
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffer Saline
PBSTw	PBS Tween-20
PBT	PBS Triton X-100
PCOLCE	type-1 ProCOLlagen C-proteinase Enhancer protein
PCR	Polymerase Chain Reaction
PFA	ParaFormAldehyde
PLP	ProteoLipid Protein
POD	PerOxiDase
PrP	Prion Protein
PS	Presinilin
qPCR	quantitative PCR
qRT-PCR	Real Time qPCR
R	Receptor
RANK	Receptor Activator of NFκB
RG	Radial Glia
rhSFRP1	recombinant human SFRP1
RNA	RiboNucleic Acid
SASP	Senescence-Associated Secretory Phenotype
Sfrp	Secreted Frizzled Related Protein
sTREM	soluble TREM2
SVZ	Sub-Ventricular Zone
TBS	Tris-Buffered Saline
TBST	TBS Tween-20
TGFβ	Transforming Growth Factor β
TIMP	Tissue Inhibitor of MetalloProteases
TioS	Thioflavin S
TLR	Toll-Like Receptor
TNFα	Tumour Necrosis Factor α
TREM2	Triggering Receptor Expressed on Myeloid cells 2

INTRODUCTION

The Central Nervous System (CNS) might represent the most complex entity in existence. It derives from the embryonic neural plate, which subsequently folds to form the neural tube. The neural tube is then patterned to form the eyes, brain and spinal cord. Within the CNS, numerous cell types behave in concordance to maintain its integrity and functions, intermingled in between a well-organised network of neurons, glia and endothelial cells in contact to each other. In order to preserve neural homeostasis, glial cells regulate diverse immune processes to secure proper functioning of the CNS. When dysfunctional, detrimental immune processes favour the progression of neurological disorders, interfering with motor control, perception, learning and memory, which lead to the incapacitation of the individual. Furthermore, aging represents a risk factor for many neurodegenerative events. In the developed world, the increasing cohort of aged individuals make neuroinflammation an important therapeutic target to make long lives worth living.

1. Immune response in the Central Nervous System

For a long time the CNS and the immune system have been thought to behave as two independent and isolated systems (Medawar, 1948). But nowadays, this dogma appears to be no longer valid as there is growing evidence that the two systems are interconnected and synergize to regulate CNS homeostasis. Indeed, the activation and infiltration of immune mediators into the CNS has been widely described under homeostatic and pathological conditions and alterations of this crosstalk have been correlated to the onset and progression of neurodegenerative diseases and aging (Lucin & Wyss-Coray, 2009).

This dogma was based on the idea that the CNS was completely isolated from the immune system and therefore this absolute isolation made the CNS considered as immune privileged, suggesting that immune processes were of no importance in the CNS. The Blood-Brain Barrier (BBB) was the only responsible for this isolation, shielding the CNS from the entry of infectious agents. However, CNS immune privilege is now known to be relative and the innate immune system has been shown to be active in the CNS. Immune system functions are mediated by a complex crosstalk that involves every cell type within the CNS (Lampron et al., 2013). More precisely, several observations have described an active contribution of the BBB to the immune response. Importantly,

an organised modulation of the permeability of the BBB allows peripheral immune cells to cross the intact BBB (Carson et al., 2006), and afterwards the BBB modulates the activity of infiltrated cells (Ifergan et al., 2008).

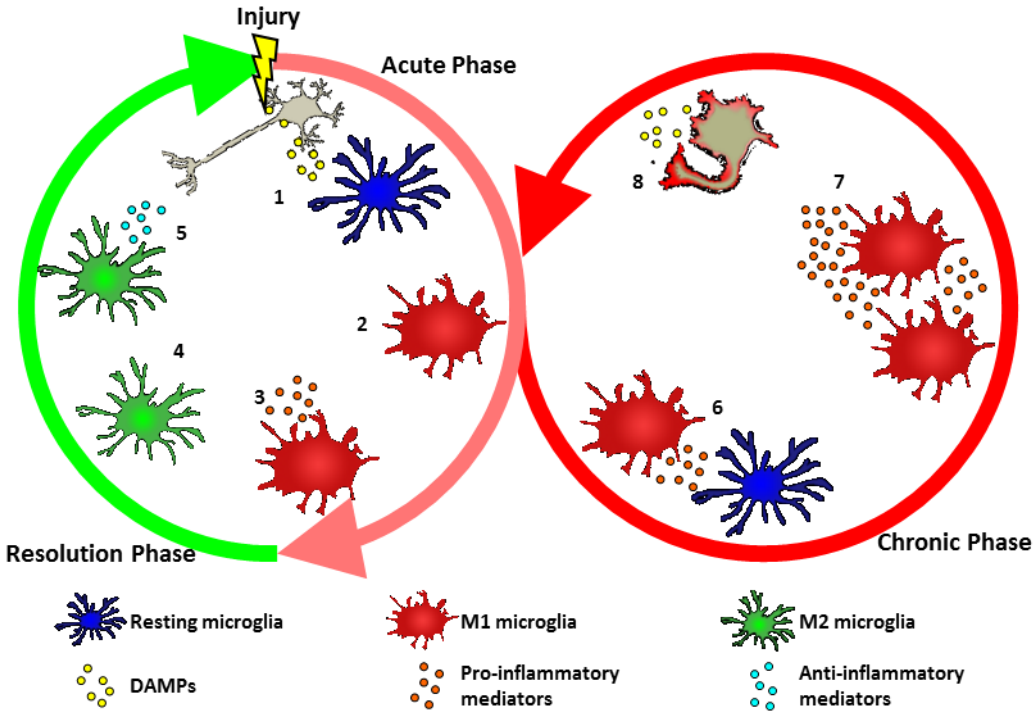


FIG.1: Schematic representation of neuroinflammatory events. The alteration of CNS homeostasis by either injury, that induces the release or exposure of DAMPs (1), or the presence of infectious agents, recognised as PAMPs, is detected by immune mediators, mostly microglial cells. This recognition promotes the rapid activation of pro-inflammatory responses by microglial cells (2), inducing the secretion of diverse mediators of inflammation, such as cytokines, chemokines and reactive species (3). Once the disturbance is resolved, microglial cells switch their activated phenotype into an anti-inflammatory state (4). To promote healing and tissue repair, microglia starts to secrete anti-inflammatory cytokines and growth factors (5) restoring CNS homeostasis. In some pathological conditions, the inflammatory response becomes chronic increasing the response of glial cells in a vicious cycle (6). This persistent activation of glial cells exacerbates the secretion of pro-inflammatory molecules (7) that becomes neurotoxic and induce neuronal degeneration (8).

Several immune processes are initiated and regulated within the CNS to preserve and prevent disturbances of neural homeostasis (Fig.1). Glial cells, astrocytes and mostly microglial cells, mediate the innate immune processes and neuroinflammation within the CNS (Hui et al., 2016). In physiological conditions, these cells produce and secrete pro- and anti-inflammatory mediators and neurotrophic factors to maintain the correct functions of the CNS. The detection of either endogenous damage-associated molecular patterns (DAMPs) or exogenous pathogen-associated molecular patterns (PAMPs) causes the rapid and early activation of glial cells. This activation induces the production of a wide range of inflammatory mediators, such as pro- and anti-inflammatory

cytokines, chemokines and reactive species (Sochocka et al., 2016). An acute inflammatory response is necessary for the clearance of debris and tissue repair of the damaged area. After this period, a resolution phase begins, characterised by the production of anti-inflammatory mediators (e.g., IL10 and TGFβ) and the depletion of pro-inflammatory molecules (e.g., IL1β and TNFα) (Shichita et al., 2014). However, when damaging agents or dysregulated activation of glial cells persist, the acute response becomes a chronic inflammatory state, in which the magnified activation of glial cells impairs the BBB and leads to tissue degeneration (Gualtierotti et al., 2017). It has been proposed that sustained inflammatory events can contribute to aging. A systemic, low-graded, chronic inflammation, named “Inflammaging”, is considered as a hallmark of aging and a strong risk factor of many neurodegenerative disorders (Franceschi & Campisi, 2014).

2. Cellular mediators of Neuroinflammation

As mentioned above, inflammatory processes within the CNS are mediated by astrocytes and microglial cells. These cell types exert multiple functions in the CNS, including protective and restorative responses to CNS infection or injury (Ransohoff & Brown, 2012).

Astrocytes

Astrocytes are the most abundant cell type in the brain and have diverse roles controlling numerous aspects of nervous system development, plasticity and disease. Named because of their characteristic stellate shape, they were described for the first time as neuroglia by Rudolf Virchow in 1856 (Parpura & Verkhratsky, 2012). However, their morphology differs depending on the developmental stage, subtype and localization (Tabata, 2015). The presence of genetically abnormal astrocytes or of astrocytes that cannot perform their functions lead to neurodevelopmental (Sloan & Barres, 2014) o neurodegenerative disorders (Belanger & Magistretti, 2009).

Astrocytes have a neural origin and, as neurons and oligodendrocytes, are born in a temporally derived manner from subsequent divisions of neural stem cells (NSCs). The first divisions of NSCs give rise to a restricted neurogenic wave, through the direct or indirect asymmetric division of elongated NSCs called radial glial (RG) cells (Noctor et al.,

2004). In general, at the end of neurogenesis, astrogenesis starts and has its peak around postnatal day 7 (P7). During this period, RG divisions directly produce astrocytes, until the last terminal division after which RG themselves differentiate into astrocytes (Pinto & Gotz, 2007). Newborn astrocytes further divide symmetrically to expand different populations of astrocytes with diverse specific positional and morphological identities (Garcia-Marques & Lopez-Mascaraque, 2013) during the first three weeks of postnatal development (Ge et al., 2012). Astrocytes in the adult subventricular and subgranular zones of the brain, along the lateral ventricular walls and the dentate gyrus of the hippocampus respectively, have been shown to generate new neurons and glial cells and are therefore considered the NSCs of the adult brain (Kriegstein & Alvarez-Buylla, 2009).

The astrocytes are the only CNS cell type that contains and metabolizes glycogen, thus representing the largest CNS energy storage, which is fundamental to support neuronal function. Astrocytes also regulate postnatal angiogenesis and the formation and permeability of the BBB by directly interacting with blood vessels through their perivascular end-feet (Obermeier et al., 2013). Astrocytes influence the environmental pH, ion homeostasis and regulate oxidative stress and blood flow (Takano et al., 2006). Importantly, astrocytes modulate neuronal conductivity and synaptic plasticity thanks to their close bidirectional interaction with neurons, forming the so-called tripartite synapses (Araque et al., 1999) and to their recycling of neurotransmitters (Singh & Abraham, 2017). Astrocytes also play critical roles in synapse formation, maturation and elimination (Clarke & Barres, 2013; Chung et al., 2013).

Upon perturbation of CNS homeostasis, astrocytes respond quickly in a severity and context-specific manner. They progressively modify their morphology, antigenicity and functions. This reactive state has been reported to induce both potentially beneficial and detrimental effects. The prototypical marker for their immunohistochemical identification is the glial fibrillary acid protein (GFAP). Detectable in many astrocytes throughout the CNS, GFAP expression increases as a sensitive and reliable marker that labels reactive astrocytes in response to CNS injuries (Sofroniew & Vinters, 2010). Other aspects of reactive astrogliosis are cell hypertrophy, proliferation and secretion of pro- and anti-inflammatory mediators. Astrocyte can secrete a large number of cytokines and

chemokines such as interleukin (IL)1β , IL6, IL8, IL10, IL17, IL27, TNFα, TGFβ, IFNγ, IFNβ, CCL2, CCL3, CCL5, CXCL10, and CXCL12 (Qin & Benveniste, 2012). In response to severe insults, astrocytes protect the CNS forming a so-called astrocytic scar. The formation of this scar facilitates BBB repair, reduces edema after trauma, stroke or hydrocephalus, stabilises extracellular matrix from excitotoxicity and oxidative stress, and limits the spread of infiltrated cells or infectious agents from areas of damage or disease into the healthy parenchyma (Sofroniew, 2009). In addition, astrocytes secrete ATP to induce a rapid response of microglia to local injury (Davalos et al., 2005).

Microglia

Microglial cells were first described by Pío del Río-Hortega in 1919, among the cells identified as the "third element" by Santiago Ramón y Cajal (Sierra et al., 2016). Microglial cells are the CNS's resident macrophages and the only cell type present in the CNS parenchyma, with the exception of vascular cells, that do not have a neural origin (Prinz & Priller, 2017). However, gene-expression profiles revealed that microglia differ considerably from other tissue-resident macrophages (Gautier et al., 2012). Microglia initiate, participate and regulate many important events of CNS development, its normal homeostasis and its pathological conditions (Ransohoff & Cardona, 2010).

Although the myeloid origin of microglial cells has been widely accepted, the real identity of microglial progenitors has been a matter of debate until recently (Tremblay et al., 2015). Fate-map studies have demonstrated that microglia derive from yolk-sac macrophages that colonise the neuroepithelium at E9.5 (Ginhoux et al., 2010). The first gradual increase of microglial cells between E10 and E14 turns into a massive proliferation around E17.5, accumulating in the choroid plexus primordium and ventricles (Swinnen et al., 2013). After birth, the pool of microglial cells continues to rise from 2% in newborn brain up to about 10% in the P14 brain (Alliot et al., 1999). During this period, microglial cells scatter throughout the brain, undergoing morphological (Reemst et al., 2016) changes from amoeboid proliferating and migrating microglia into ramified parenchymal microglia. These modifications are associated with transcriptional changes (Matcovitch-Natan et al., 2016). Under physiological conditions, microglia locally self-renew slowly throughout life (Ajami et al., 2007), coupling proliferative and apoptotic processes to maintain their number in the adult brain (Askew et al., 2017).

During postnatal development, microglia play an important role in pruning of synapses (Paolicelli & Ferretti, 2017) and phagocytosis of apoptotic newborn neurons (Marin-Teva et al., 2011). This close interaction with neurons persist in the adult CNS, where microglia remove apoptotic neurons (Neumann et al., 2009) and function as dynamic regulators of synaptic plasticity (Wu et al., 2015), thus actively contributing to learning and memory (Tremblay et al., 2010). In addition, microglia support neurons by releasing neurotrophic factors (Heneka & O'Banion, 2007), and are required for appropriate maturation of excitatory synapses (Salter & Beggs, 2014). All these processes are supported by microglia remarkable ability of constantly surveying the entire CNS (glia, blood vessels and neurons) with their highly motile processes typical of the ramified microglia (Nimmerjahn et al., 2005), that paradoxically have been commonly referred as resting microglia.

Microglial cells represent the first line of defence when CNS homeostasis is challenged by a broad range of abnormal conditions, including injury, infection, ischemia, toxic insults, trauma as well as different chemicals, cytokines, abnormally folded or aggregated neurotoxic proteins (Luo & Chen, 2012). The microglial cell surface is equipped with numerous transporters, channels and receptors for neurotransmitters, neuro-hormones, neuromodulators, as well as wide range of receptors to detect PAMPs or DAMPs within the CNS (Kettenmann et al., 2011). These include Toll-like receptors (TLR), NOD-like receptors (NLR), and receptors for nucleic acids. In addition, they express several families of receptors that enable phagocytosis of apoptotic cells, protein aggregates and lipoprotein particles, such as ApoER. Microglia also capture and endocyte immune complexes and complement-opsonized protein complexes through Fc receptors and complement receptors. Microglia express chemokine receptors (e.g., CX3CR1) and immune receptors that regulate activation processes, such as members of the immunoglobulin superfamily that deliver either activating (e.g., triggering receptor expressed on myeloid cells 2, TREM2) or inhibitory signals (e.g., CD200R). Microglia activity is also regulated by receptors of pro- and anti-inflammatory cytokines, produced in the CNS by glial cells or that reach the CNS from the circulation (Colonna & Butovsky, 2017).

Upon any type of disturbance of the CNS, resting microglia become activated, proliferate, change cell morphology and migrate to the damaged area (Ransohoff & Perry, 2009). Microglial activation is a complex process that may lead to a wide spectrum of activations typically categorised as “classical M1” or “alternative M2” activation (Fig.2), depending on the microenvironment and triggering factors (Heneka et al., 2014).

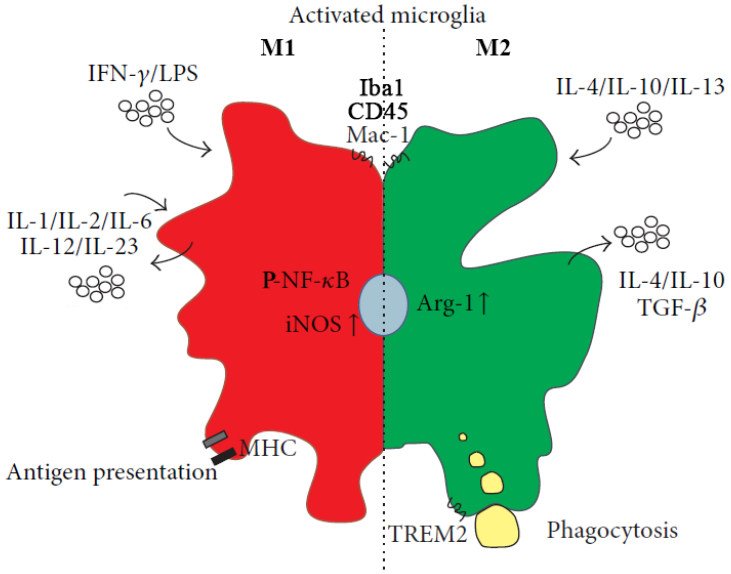


FIG.2: Polarization of microglial cells. Microglia are activated by diverse stimuli that trigger a wide range of responses of microglial cells. Within the spectrum of activation states in which microglia evolve, microglial phenotypes have been typically divided in the pro-inflammatory M1 status and the anti-inflammatory M2 status. These phenotypes have been widely characterised. While IFNγ/LPS promote the M1 status, IL4/IL10/IL13 or apoptotic cells induce the M2 status. M1 microglia secrete wide range of pro-inflammatory cytokines such as IL1β/IL6/TNFα and overexpress MHC class II and Fc receptors to mediate interactions with infiltrated cells. M2 microglia secrete instead trophic factors as TGFβ and anti-inflammatory cytokines such as IL4/IL10 and promote phagocytic activity mediated by TREM2. Figure adapted from Goldmann & Prinz, 2013.

M1 activation initiates the defensive response and is induced by TLR and IFNγ signalling, resulting in the expression of pro-inflammatory mediators and enhanced microbicide capacity. M1 microglia overexpress MHC class II, Fc receptors, integrins (CD11b), and pro-inflammatory cytokines and chemokines, like IFNγ, IL1β, TNFα, IL6, IL18, IL12, IL23 and CCL2. When sustained, M1 activation may cause collateral neurotoxicity (Du et al., 2016). To stop the inflammatory phase of classically activated microglia, a switch between the activated states is necessary (Perry et al., 2007). Microglial M2 activation is induced by IL4, IL13, IL10, TGFβ, and detection of apoptotic cells. M2 microglia triggers anti-inflammatory and healing activities to resolve inflammation and support tissue repair and reconstruction. These different activities have been proposed to distinguish between alternatively activated subtypes of

microglia (Colton, 2009). The M2 phenotype is characterised by enhanced phagocytic activity, induced expression of arginase 1 and secretion of anti-inflammatory cytokines, such as IL4, IL10, IL13, and TGFβ, and growth factors, such as insulin-like growth factor (IGF1), fibroblast growth factor (FGF), colony-stimulating factor (CSF1), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial derived neurotrophic factor (GDNF) (Boche et al., 2013). The distinction of activated microglial states into M1/M2 is a simplification since M1 and M2 represent the extremes of a range of activated phenotypes.

3. Neuroimmune Regulators

As mentioned before, the transition of microglial cells from a surveying phenotype towards an activated state is closely regulated by several extrinsic factors detected by the wide range of receptors present in the microglial cell membrane (e.g., CX3CR1, CD200R, and TREM2; Fig.3). The interaction of this recognition system is required for the proper maintenance of CNS homeostasis and the fine tuning of the microglial response (Linnartz & Neumann, 2013). Minor changes of these signalling cascades lead to major dysregulation of microglial activity and to damage neuronal integrity and function, and, as result, to develop CNS pathologies (Kierdorf & Prinz, 2013).

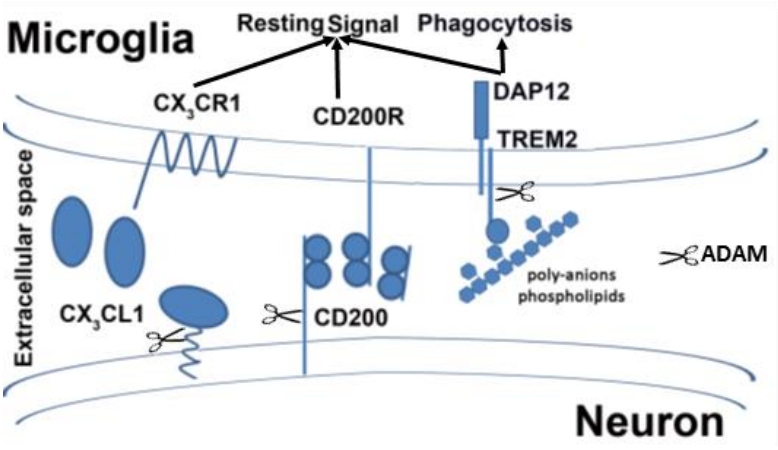


FIG.3: Immune regulators of microglial response. Microglial activation is widely controlled by signals detected by membrane receptors that trigger inhibition or activation signals in microglia to self-maintain under resting conditions. Microglial inhibitory receptors CX3CR1 and CD200R sense ligands expressed or released by neurons. CX3CL1 and CD200 are present in the surface of healthy neurons, and loss of interaction is a signal of neuronal damage. TREM2 sense CNS environment by recognising purines, phospholipids and lipoproteins that mimic neuronal injury. TREM2 activation triggers phagocytic and anti-inflammatory responses in microglial cells. Components of the three sensors signalling are processed by ADAM10, represented by scissors in the diagram. Figure adapted from Kierdorf & Prinz, 2013.

CX3CR1

CX3CR1 is the receptor of the chemokine CX3CL1, also known as fractalkine or neurotactin, and represents the only member of a monogamous ligand/receptor system of the CX3C chemokine family (Ransohoff & El Khoury, 2015). The expression of CX3CR1 in the CNS is restricted to the microglial population (Jung et al., 2000), while the ligand can be found in the neuronal membrane, as one of the two unique membrane-bound chemokines, or in a secreted form (Hughes et al., 2002). The release of the chemokine domain is mediated by ADAM10 (Hundhausen et al., 2007). CX3CR1/CX3CL1 signalling plays a critical role regulating diverse microglial functions during physiological conditions, modulating microglial surveillance and interaction with neurons implicated in the maturation, activity and plasticity of neuronal connectivity (Paolicelli et al., 2014). Under healthy conditions, high levels of secreted CX3CL1 have been found in the CNS parenchyma, but if reduced promote microglial neurotoxicity (Cardona et al., 2006). Furthermore, impaired signalling have been involved in the development of cognitive impairment (Rogers et al., 2011) and chronic inflammatory diseases (Clark et al., 2011), and the mediation of recruitment of immune cells in autoimmunity (Hertwig et al., 2016).

CD200R

CD200R is another inhibitory receptor expressed in microglial cells (G. J. Wright et al., 2000) and recently observed also in activated astrocytes (Hernangomez et al., 2016). Its ligand, CD200 also known as OX2, is mainly expressed by neurons and, to a lower extent, by astrocytes and oligodendrocytes (Koning et al., 2009). The interaction between CD200 and CD200R keeps microglia in a resting state (Lyons et al., 2007), and most importantly, induces the return of pro-inflammatory activated microglia to this state (Walker & Lue, 2013). Both proteins are characterised by two immunoglobulins domains, but they differ in that CD200 lacks the longer cytoplasmic tail of CD200R with an immune-receptor tyrosine-based inhibition motif (ITIM) domain (Barclay et al., 2002). Deficient interaction or expression of this immune-regulatory system has been reported in AD (Walker et al., 2009) and demyelinating diseases (Hernangomez et al., 2012) leading to chronic inflammation and contributing to aging (Hernangomez et al., 2014).

The release of a functionally active soluble form of CD200 from the plasma-membrane has been recently reported, as a consequence of an ADAM protease mediated shedding, by ADAM10, 17 and 28. This soluble form seems to have detrimental immunosuppressive effects in patients with leukaemia (Wong et al., 2016) but, at the moment, there is no reported information about the impact of the CD200 soluble form of in the brain.

TREM2

The Triggering Receptor Expressed on Myeloid cells 2 (TREM2) is a transmembrane glycoprotein with an extracellular single immunoglobulin domain expressed only by microglia in the CNS (Colonna & Wang, 2016). TREM2 binds poly-anions, such as bacterial lipopolysaccharides (Y. Wang et al., 2015), phospholipids and lipoproteins such as ApoE (Yeh et al., 2016). Ligand-TREM2 interaction transmits intracellular signalling through the immune-receptor tyrosine-based activator motif (ITAM) of DAP12, with which TREM2 is associated through their transmembrane regions, promoting proliferation and survival (Otero et al., 2009), phagocytosis of apoptotic cells (Hsieh et al., 2009), and attenuates microglial pro-inflammatory signalling (Turnbull et al., 2006).

Rare variants of TREM2 have been associated with an increased risk of developing sporadic AD (Guerreiro et al., 2013; Jonsson et al., 2013). Even more rare mutations in TREM2 or DAP12, that impair signalling, cause Nasu-Hakola disease, an inherited form of dementia (Paloneva et al., 2002). Furthermore, deficiency of TREM2 in a mice model of AD present less clustering of microglia surrounding the Aβ plaques (Jay et al., 2015), which facilitates Aβ diffusion and consequently toxicity (Y. Wang et al., 2016). Recent studies have also demonstrated that TREM2 is processed at the microglial cell surface by ADAM10 and ADAM17 sheddases, generating a soluble form (Kleinberger et al., 2014). This soluble TREM2 can be detected in the cerebrospinal fluid, acting as a marker of microglial activation that correlates with neuronal injury markers (Suarez-Calvet et al., 2016). Importantly, latest data indicate that soluble TREM2 may have its own function triggering survival and pro-inflammatory responses, leading to microglial activation (Zhong et al., 2017).

4. Models of Neuroinflammation

The roman physician Aulus Celsus originally defined the term “inflammation” as a process of “tumor, rubor, calor et dolor” (Celsus, 1478). This four cardinal sings imply extravasation of the adaptive immune response mediators T and B-lymphocytes. Notably, whereas this feature is seen in bacterial and viral infections and in autoimmune diseases (e.g., Multiple Sclerosis, MS) of the CNS (Aguzzi et al., 2013), the term “neuroinflammation” has gradually expanded to include conditions in which Celsus’ s cardinal signs are not present. Neurodegenerative diseases, such as AD, are now considered neuroinflammatory conditions, in which proliferation and activation of glial cells are well-established hallmarks (Ransohoff, 2016).

Lipopolysaccharide induced Neuroinflammation

One of the typical approximations to study the immune system has been based on the use of the endotoxin lipopolysaccharide (LPS). LPS, the best-characterised microbial PAMP, is a major component of the outer membrane of Gram-negative bacteria and its recognition induces a robust inflammatory response by phagocytic cells (S. D. Wright, 1999). Cell wall components of Gram-negative and Gram-positive bacteria stimulate cytokine production, activating Toll-like receptors (TLRs) and pro-inflammatory signalling (Nguyen et al., 2002). While TLR2 recognises Gram-positive bacteria, TLR4 is critical for the recognition of LPS (S. J. Lee & Lee, 2002). Binding of LPS to TLR4 induces the stimulation of nuclear factor-kappaB (NFκB) signalling pathway and cytokine synthesis (Allan et al., 2005).

Systemic injection of LPS is recognised by TLR4-expressing microglia in the circumventricular organs and choroid plexus, inducing the expression of TNFα that spreads the pro-inflammatory signal through the brain parenchyma. Similarly, LPS injected directly into the brain induces robust and transient microglial M1 activation and expression of pro-inflammatory molecules, such as cytokines, chemokines and complement system proteins, by a TLR4-dependent mechanism (Rivest, 2009). LPS locally-injected stimulates microglial cells directly through the TLR4-NFκB pathway, whereas activation of parenchymal microglia after a systemic LPS challenge depends on TNFα-induced signalling (Nadeau & Rivest, 2000). Although TLR4 expression by

astrocytes and neurons has been reported (Bowman et al., 2003), the response to LPS is completely dependent on the presence of functional microglia (Holm et al., 2012).

Neuroinflammation associated with CNS autoimmunity

Experimental Autoimmune Encephalomyelitis (EAE) is the most commonly used experimental model to study the prototypical inflammatory demyelinating disease of the CNS, i.e., Multiple Sclerosis (MS). MS is a degenerative autoimmune disease with a strong inflammatory component that attacks CNS myelin. From a clinical point to view, MS exhibits a relapsing and remitting pattern of neurological deficits that can resolve completely or leave residual disabilities, and occasionally derives in a continuous progressive disease (Lassmann et al., 2012). The pathological features of MS are the disruption of the BBB accompanied by an infiltration of peripheral macrophages and lymphocytes, and an intrinsic gliosis, that ultimately results in glial scar formation surrounding axonal degeneration and demyelination sites, which is known as sclerotic plaque, the principal hallmark of MS (Pérez-Cerdá et al., 2016). It is controversial if macrophage and lymphocyte infiltration (Barnett & Prineas, 2004), believed to be the main trigger of CNS damage, is a cause or a consequence of the degeneration of neural tissue (Stys et al., 2012). The induction of EAE is achieved by animal immunization and therefore it is of no help to solve this controversy (Gran et al., 2008).

Most of the MS features, including paralysis, weight loss, demyelination, inflammation and BBB disruption, are observed during EAE (Bennett et al., 2010). EAE is typically induced by the sensitization of the innate immune system to different myelin derived proteins such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), or proteolipid protein, injected together with an adjuvant, usually complete Freund's adjuvant, and pertussis toxin to relax the BBB (Gao & Tsirka, 2011). The pathological lesions vary among strains and type of immunization, with monophasic or sustained form of EAE. This depends on the presence or reduced resolution of multifocal demyelination and infiltration of macrophages and CD4 positive T-cells (Constantinescu et al., 2011). Macrophage infiltration strongly correlates with EAE progression to severe disease, and disappearance of macrophage from the CNS lead to symptom remission and possible recovery (Ajami et al., 2011).

In response to passive immunization, microglia become activated and proliferate, this neuroinflammatory process have been implicated in the onset and severity of EAE clinical signs (Ding et al., 2014). During the onset and peak of EAE, microglia phagocytize myelin, triggering the release of cytokines and chemokines that induce the disruption of BBB integrity and the recruitment of macrophages and lymphocytes, that can be activated by myelin presentation in MHC class II of microglial cells, acting as Antigen Presenting Cells (Goldmann & Prinz, 2013). In addition, microglial cytokine release has an important role in the regulation, proliferation and differentiation of the infiltrated T-cells (Fletcher et al., 2010). Importantly, infiltrated macrophages and resident microglia seems to have different roles in the course of EAE. Infiltrated macrophages are highly inflammatory and demyelinating, whereas activated microglia appear to clear debris (Yamasaki et al., 2014). Microglial overexpression of TREM2 increases the phagocytic clearance of myelin debris, leading to improved tissue regeneration and to reduction of the severity of the clinical symptoms (Takahashi et al., 2007).

Neuroinflammation associated with Neurodegeneration

Alzheimer's disease is a neurodegenerative disease characterised by the accumulation of amyloidβ (Aβ) containing plaques and Tau-positive neurofibrillary tangles, accompanied by gliosis and neuronal loss (Alzheimer et al., 1995). Other morphological features of AD include cerebrovascular amyloid angiopathy and major synaptic alterations (Crews & Masliah, 2010). How AD develops is still unclear although there are several well-established risk factors such as aging, diabetes, vascular alterations, and a long etcetera. There are also several hypotheses on the possible triggers. One of them, the amyloid hypothesis, links abnormal Aβ peptide aggregation and accumulation with the other hallmarks of the disease. Toxic Aβ peptides are generated by the action of two proteases: β-secretase (BACE1) and γ-secretase complex (composed by PS1/2, Nicastrin and APH1), which sequentially cleave the amyloid precursor protein (APP). Aβ deposition linearly and causally leads in a progressive cascade to Tau pathology, neuroinflammation, synaptic dysfunction, neuronal loss and, ultimately, dementia (Hardy & Selkoe, 2002). In the past, many clinical trials designed to reduce Aβ levels show no efficacy. However, a recent clinical trial that selectively targeted Aβ oligomers, the more toxic form of Aβ, showed some improvement,

providing support to this hypothesis. Indeed, treatment with antibodies against oligomeric Aβ reduced plaque burden and slowed the progression of cognitive decline in AD patients (Sevigny et al., 2016).

The central role of Aβ accumulation in the pathogenesis of AD was revealed by the discovery of mutations in the *APP or Presinilin* genes leading to altered APP processing in familial forms of AD (Hardy & Allsop, 1991). These mutations have been subsequently exploited to generate mouse models for AD (LaFerla & Green, 2012). One of the most used is the APP;PS1 mouse line. These mice overexpress, under the control of the PrP promoter, the human APP allele carrying the so-called Swedish mutation (K594M/N595L) and a human PSEN1 allele containing a deletion of exon 9 (Jankowsky et al., 2004).

The linearity of the amyloid hypothesis is nevertheless very controversial. The initial causality has been modified (Selkoe & Hardy, 2016) to enhance the importance of Aβ clearance impairment, that involve the expression of ApoE4 allele (Castellano et al., 2011), and the misbalance of APP processing, leading to accumulation of longer Aβ products (Aβ42/43) that are highly self-aggregating. The neuron-centric view has been expanded towards the contribution of different cell-types, and their interactions, that have been involved in the gradual evolution of the disease, trying to explain different confusing steps of the cascade, such as the silent incubation period of Aβ accumulation and the link between Aβ and neurotoxicity (De Strooper & Karran, 2016).

The role of neuroinflammation in the pathogenesis of AD has been intensively investigated (Heneka et al., 2015). The prototypical hallmarks of AD, hyper-phosphorylated Tau and oligomeric and fibrillary Aβ, are highly immunogenic and trigger the activation of microglial cells and astrocytes, inducing the secretion of pro-inflammatory cytokines (Grubman et al., 2016). In the brain of AD patients, reactive astrocytes occupy peri-plaque positions, surrounding Aβ deposits in a manner reminiscent of glial scarring (J. J. Rodriguez et al., 2009). Furthermore, there is a chronic increase of the levels of pro-inflammatory cytokines (Brosseron et al., 2014). Nevertheless, anti-inflammatory strategies seem to have unexpected negative effects on Aβ processing and cognition (Chakrabarty et al., 2015). This demonstrates the complexity of neuroinflammation associated to neurodegeneration since in some

situations it can be beneficial and in other detrimental. Importantly, sporadic AD risk genes are predominantly expressed by microglia. This indicate that genetically impaired microglia may represent a risk for AD, further reinforcing the relevance of neuroinflammation in AD (Skene & Grant, 2016). These genes are mostly involved in the regulation of neuroinflammation and phagocytosis and include TREM2, CR1, CD33 and ApoE4 (Villegas-Llerena et al., 2016). Moreover, it seems that the physiology of microglial cells is impaired in the brain of AD patients likely because of chronic stimulation or loss of function (Mosher & Wyss-Coray, 2014). This dysfunction contributes to early synapse loss in AD, in part because complement-mediated synapse pruning does not properly work (Hong et al., 2016).

Importantly, taking into account that aging is the greatest risk factor for AD, a recent time-course study has demonstrated, comparing gene expression profiles in normal and AD brains, that immune- and inflammation-associated genes were robustly upregulated in aged brains compared to a modest response in AD patients (Cribbs et al., 2012). This demonstrates the critical involvement of neuroinflammatory processes in AD development and progression.

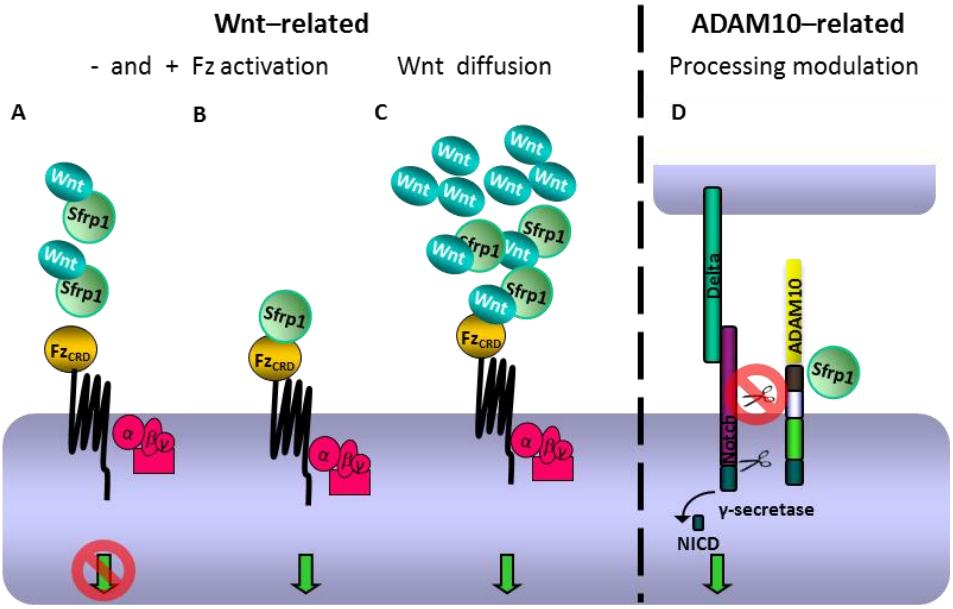
5. Secreted Frizzled-Related Protein 1

Secreted-Frizzled-Related-Proteins (Sfrps) compose a family of soluble factors with five members in mammals (Sfrp1-5). These highly diffusible proteins have been widely studied and characterised as modulators of Wnt signalling, an extensively used signalling pathway that mediates cell-cell communication in development and adult tissue homeostasis (Bovolenta et al., 2008). The different members of the family were independently identified in two different contexts: related to early embryonic development (Leyns et al., 1997) and in the modulation of apoptosis (Melkonyan et al., 1997). This family received its name because the N-terminal region of the proteins presents high sequence homology with the extracellular Wnt binding domain of the Frizzled receptors (Hoang et al., 1996). The proteins are composed of two independently folded domains. The domain similar to that of Frizzled receptor is called Cysteine Rich Domain (CRD), owing to the ten conserved cysteine residues that conform a pattern of disulphide bridges. The C-terminal domain contains a Netrin-related motif (NTR). In the closely related Sfrp1/2/5, this domain is characterised by segments of positively charged

residues and six cysteine residues that form three disulphide bridges (Chong et al., 2002). This domain shares conformational similarities with a number of proteins, including the axon-guidance protein netrin1, tissue inhibitors of metalloproteases (TIMPs), type-1 procollagen C-proteinase enhancer proteins (PCOLCEs) and complement proteins (Banyai & Patthy, 1999).

Roles of Sfrp1 as regulator of cell-cell communication

Initial studies associated Sfrp3, the funding member of the family originally identified as FrzB, with Wnt signalling because of its ability to bind Wnt ligands (Fig.4A) and prevent signalling activation (S. Wang et al., 1997), thus acting as Wnt antagonists (Kawano & Kypta, 2003). This idea is supported by some aspects of the phenotype of the knockout mice for Sfrp1, Sfrp2 and Sfrp5, which are partly redundant (Satoh et al., 2008). In some cases instead, Sfrps have been shown to have opposite effects (Melkonyan et al., 1997). Direct interaction between Wnt and the CRD domain of Sfrps was postulated to be necessary and sufficient to bind and inhibit Wnt (Lin et al., 1997). However, other studies have shown that the NTR of Sfrp1 can also bind to Wnt (Lopez-Rios et al., 2008; Uren et al., 2000) and there is evidence that both domains are likely required for optimal Wnt inhibition (Bhat et al., 2007). Sfrp-Wnt interaction inhibit both canonical βcatenin-dependent and non-canonical pathway (Mii & Taira, 2011), in distinct processes such as specification of the antero-posterior axis, regulation of gastrulation movements, and patterning of the neural tube, somitogenesis, vascular endothelium and heart formation (Cruciat & Niehrs, 2013).



Beside inhibition of Wnt signalling, Sfrps have other Wnt-dependent and independent roles, as shown by several studies from different laboratories, including ours (Esteve & Bovolenta, 2010). Indeed, Sfrps can promote or suppress Wnt signalling depending on their concentration and cellular context (Xavier et al., 2014). Direct interaction between Sfrp1 and Frizzled receptors can induce the activation of the Wnt non-canonical pathway (Fig.4B), providing axon-guidance information to retinal ganglion cell axons (J. Rodriguez et al., 2005). Sfrp-Wnt interaction can also promote Wnt diffusion (Fig.4C) in both Xenopus gastrulation (Mii & Taira, 2009) during optic cup formation (Esteve et al., 2011b). Besides this Wnt signalling related functions, Sfrps can regulate other pathways. Sfrp1 interaction with RANKL, a member of the TNF family, prevents its binding to the activator of NFκB receptor (RANK), inhibiting osteoclast formation (Hausler et al., 2004). In addition, the CRD of Sizzled, a Sfrp not expressed in mammals, binds to BMP1/tolloid, impairing its metalloprotease activity required to regulate a BMP signalling inhibitor, thereby regulating the pathway (H. X. Lee et al., 2006). Our laboratory demonstrated that Sfrp1 interacts and negatively modulates the activity of the A Disintegrin and Metalloprotease transmembrane protein ADAM10 (Fig.4D). This modulation interferes with ADAM10-mediated shedding of multiple substrates including N-cadherin, L1 and Notch, during retinal and cortical neurogenesis, adult brain homeostasis (Esteve et al., 2011a) and visual pathway establishment (Marcos et al., 2015).

FIG.4: Sfrp1 are multifunctional regulator of cell communication. Schematic representation of the different mechanisms by which Sfrp1 regulates embryonic development and tissue homeostasis. A) Sfrp1 act as Wnt antagonist, inhibiting the signalling cascade by ligand sequestration. B) Sfrp1 promotes Wnt signalling by direct interaction with Frizzled receptor. C) Sfrps bind and promote the diffusion of Wnt ligands. D) Sfrp1 binds and negatively modulates the activity of the metalloprotease ADAM10 (D), thereby interfering with the shedding of multiple substrates.

Roles of Sfrp1 in pathogenesis

Wnt signalling regulates diverse processes during embryonic development including cell proliferation, patterning and fate determination (Clevers & Nusse, 2012). Mutations or abnormal expression of components of the Wnt pathway have been associated with tumorigenesis (Klaus & Birchmeier, 2008). In line with their function as inhibitors of Wnt signalling, Sfrps seem to act as tumour suppressors. Loss or downregulation of Sfrps expression, produced by allelic loss or promoter hyper-methylation, have been reported in a variety of invasive carcinomas (Surana et al., 2014) and Sfrp1 promoter hyper-methylation is an epigenetic marker for cancer detection and prognosis (Bovolenta et al., 2008).

Sfrps have been also implicated in other pathologies (Esteve & Bovolenta, 2010). For example, Sfrp1 expression is strongly increased in the retinas of patients with retinitis pigmentosa, a degenerative disease characterised by the progressive loss of photoreceptors (Hackam, 2005). Upregulation of Sfrp1 in the eye is associated with elevated intraocular pressure, leading to glaucoma, perhaps inhibiting the Wnt pathway (W. H. Wang et al., 2008). Outside the CNS, Sfrp1 overexpression contributes to the pathogenesis of periodontitis (C. H. Li & Amar, 2007), rheumatoid arthritis (Walsh et al., 2009) and lung emphysema (Foronjy et al., 2010), diseases that are all associated to chronic inflammation.

With the exception of the retina, there is no description of CNS pathologies with a direct relationship with Sfrp1 although the expression of diverse components of the Wnt signalling pathway are disrupted in AD (Godoy et al., 2014), leading to an exacerbated GSK3β activity that may represent a link between Aβ plaques and Tau neurofibrillary tangles (Llorens-Martin et al., 2014). Furthermore, dysfunction of Wnt signalling has been implicated in Aβ-mediated synaptic loss (Purro et al., 2014). The implication of Wnt ligands in inflammatory processes is controversial. Wnt3a (Halleskog et al., 2011) and Wnt5a (B. Li et al., 2011) might have pro-inflammatory properties but can also counteract LPS-induced pro-inflammatory response (Halleskog & Schulte, 2013) and induce an anti-inflammatory response (Di Liddo et al., 2015).

Implication of Sfrp1 in Alzheimer's Disease

ADAM10 is a constitutive transmembrane α-secretase highly active in the brain (Jorissen et al., 2010). ADAM10 sheds a wide range of substrates (Weber & Saftig, 2012) involved in the regulation of diverse processes in CNS development or degeneration, such as neurogenesis, axon-guidance, synaptogenesis and neuroinflammation (Saftig & Bovolenta, 2015). Among them, one of the most studied ADAM10 substrates is APP (Huovila et al., 2005).

APP is expressed in many tissues but particularly concentrated at the neuronal synapses. As mentioned above, APP is a protein widely accepted as a key factor in the onset and development of AD (Hardy & Selkoe, 2002). APP is processed by two alternative proteolytic pathways. In the so-called amyloidogenic pathway, APP is sequentially cleaved by β and γ-secretases (BACE and PS1) to generate Aβ peptides that aggregate and accumulate in Aβ plaques (Y. W. Zhang et al., 2011). APP can be also processed through an alternative "non-amyloidogenic pathway" (Kuhn et al., 2010). In this case, APP is first cleaved by the α-secretase ADAM10 within the Aβ peptide sequence, precluding the formation of Aβ peptides (Lichtenthaler, 2011). This alternative processing leads to the release of a soluble N-terminal extracellular domain known as APPsα, which has been reported to have neurotrophic and neuroprotective properties (Ring et al., 2007) and to promote adult neural stem cell proliferation (Caille et al., 2004).

APP (Yasuoka et al., 2004), ADAM10 (Demars et al., 2011) and Sfrp1(Fig.5A) are co-expressed in the sub-ventricular zone of the lateral ventricle of adult mouse brains. Consistent with the finding that Sfrp1 acts as negative modulator of ADAM10, high levels of soluble non-amyloidogenic APPsα are found in the ventricular walls of *Sfrp1* null mice (Esteve et al., 2011a), suggesting a possible involvement of Sfrp1 in the onset and progression of AD.

To test this possibility our laboratory has shown that Sfrp1 expression is increased in a mouse model of AD, the APP;PS1, accumulating in the Aβ plaques (Fig.5B) and co-localising with some surrounding reactive astrocytes (Fig.5E) and activated microglia (Fig.5H). Furthermore, genetic inactivation of *Sfrp1* in APP;PS1 mice (APP;PS1;*Sfrp1*$^{-/-}$)

increases APPsα levels and reduces Aβ plaque burden (in Fig.5, compare C with F). Most notably, APP;PS1;*Sfrp1*$^{-/-}$ mice present a decrease of the associated astrogliosis (in Fig.5, compare D with G), the virtual absence of microglia activation, and no signs of cognitive decline, traits normally observed in age matched APP;PS1 (Esteve et al., in preparation). Notably, immunohistochemical and biochemical analysis of the frontal cortex of AD patients indicate an increase and accumulation of SFRP1 in both neuritic and diffuse Aβ plaques. Consistent with these observations, a microarray-based analysis of molecular changes occurring in patients with incipient AD reported SFRP1 among the genes with increased expression in the hippocampus (Blalock et al., 2004). Remarkably, we also detected higher levels of SFRP1 in the CSF and serum samples of AD patients as compared to age matched cognitive normal individuals.

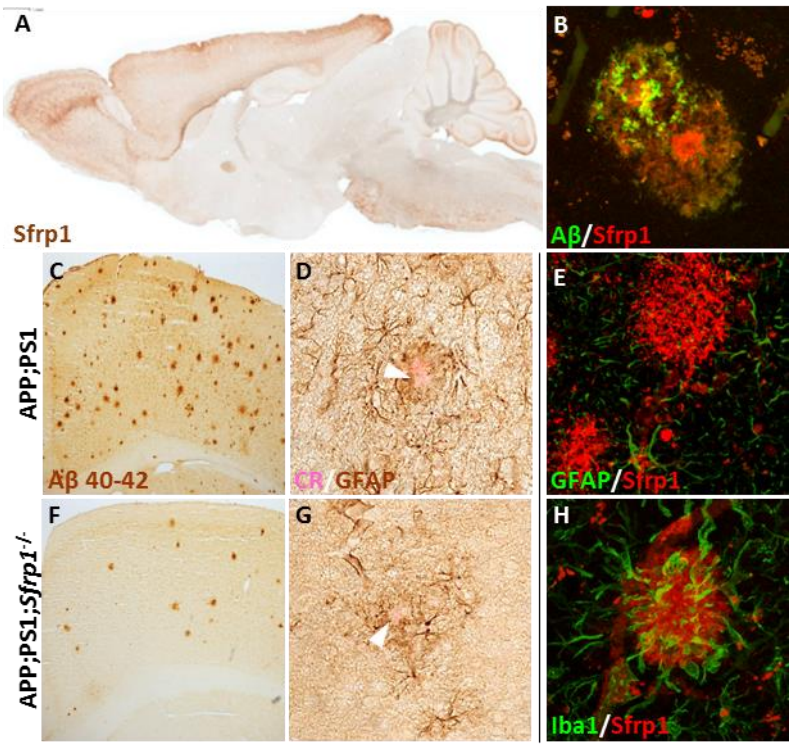


FIG.5: Sfrp1 is involved in AD pathogenesis (A) Image taken by the GENSAT webpage showing Sfrp1 expression in the cingulate cortex, rostral migratory stream, olfactory bulbs and cerebellum. Adult brains from APP;PS1 (C,D), APP;PS1;Sfrp1$^{-/-}$ (F,G) and samples from AD patients (B,E,H) were cryo-sectioned and immunostained with antibodies against Sfrp1 (B,E,H), Aβ (B,C,F), GFAP (D,E,G) and/or Iba1 (H). Sfrp1 expression is observed in AD patients Aβ plaques (C), co-localising with GFAP positive astrocytes (G) and Iba1 positive microglia (K). Genetic inactivation of Sfrp1 in a mouse AD model (F,G) reduces Aβ plaque burden (compare F with C) and associated astrogliosis (Aβ plaques are counterstained with Congo-red and marked with arrowheads, compare G with D).

Taken together, these observations demonstrate the effect of Sfrp1 as modulator of Aβ formation. The beneficial effect of decreased levels of Aβ in the absence of Sfrp1 seem higher than expected, especially when considering glial activation. This suggest that Sfrp1 could have a direct function in the regulation of inflammatory processes within the CNS. This is the question we have addressed in this Thesis.

OBJECTIVES

Sfrp1 protein is highly diffusible and acts as multifunctional extracellular regulator of cell-to-cell communication. In addition, its expression increases in diverse diseases associated with chronic inflammation. This points to a possible role of Sfrp1 in the modulation of inflammatory processes. Previous work from our laboratory has also demonstrated that Sfrp1 contributes to AD progression by modulating ADAM10-mediated processing of APP. In the course of this study we noticed that neuroinflammation was particularly low when Sfrp1 protein was absent from the brain of mouse model of AD, pointing to a possible direct implication of Sfrp1 in the regulation of brain inflammation.

We have thus undertaken this Thesis to determine whether Sfrp1 is involved in the regulation of inflammatory processes in the CNS. The relevance of this general goal relies on the growing evidence that inflammation in the CNS participates in the onset and progression of neurological disorders.

The specific objectives for this work are the following:

- Analyse the changes of Sfrp1 expression under neuroinflammatory conditions.
- Test if Sfrp1 is necessary and sufficient for the development of an inflammatory response within the CNS.
- Examine the possible mechanism of action through which Sfrp1 promotes neuroinflammation.
- Study whether Sfrp1 could be considered as therapeutic target to attenuate neuroinflammation typically observed in neurodegenerative diseases.

MATERIALS AND METHODS

1. Animals

We used newborn and adult mice of both sexes. All mice were maintained under specific pathogen–free conditions at the animal facilities of the Centro de Biología Molecular Severo Ochoa, in accordance with current national and European guidelines (Directive 2010/63/EU). All animal procedures were approved by the ethical committee of the institute and of the Comunidad Autónoma de Madrid.

Sfrp1$^{-/-}$ mice were generated by inter-cross of the *Sfrp1*$^{-/-}$;*Sfrp2*$^{+/-}$ mice in a 129/C57BL/6 background described in (Satoh et al., 2006), and back-crossed at least four times with C57BL/6J to clean the background. Wild type animals were littermates selected from heterozygous crosses. *Sfrp1*$^{-/-}$ brains present narrow ventricles and shorter but thicker cortex (Esteve et al., Submitted). APP;PS1 transgenic mice where generated as described (Jankowsky et al., 2004). Breeding pairs were kindly provided by Dr. Torres-Aleman, Instituto Cajal, CSIC, Madrid, and only male mice were used.

2. Intra-cerebral infusion

For LPS infusion into the brain parenchyma, wild type (C57BL/6J) and *Sfrp1*$^{-/-}$ male littermates of 10 weeks of age were used. Mice were anaesthetized with 4% inhaled Isoflurane (Forane, AbbVie Farmacéutica) vaporised into a sealed anaesthetic induction chamber (SurgiVet, Smiths Medical), placed into a stereotaxic apparatus (Stoelting) and anaesthesia was maintained at 2.5% in 250ml/min oxygen flow. Under aseptic conditions, a midline incision with scalpel was performded to reveal the skull and bregma area was gently cleaned. Craniotomy was performed at precise point with a 23G blunt needle, to allow injection into stereotaxic coordinates of 0.0mm anterior/posterior, -1.0mm lateral, and -1.5mm dorsal/ventral from bregma, into the top of corpus callosum (Lein et al., 2007). Vehicle (2.5µl sterile saline) or 5µg LPS (*Escherichia coli* 0111:B4; Sigma Aldrich) in 2.5µl of sterile saline were delivered using a 10µl syringe with a fine 34G needle (Hamilton). Infusion was performed with a Quintessential Stereotaxic Injector (Stoelting) at a rate of 0.5µl/min. The needle was kept in this position for an additional 5min after injection and then retrieved slowly out of the brain. Three days after infusion, when the inflammatory process reaches its peak of activation (Rivest, 2009), mice were sacrificed and processed for either biochemical or histological analysis.

In the case of lentiviral infusion, the procedure was slightly different as lentiviral particles were delivered intracerebroventricularly. First, lentiviral particles carrying GFP or SFRP1-IRES-GFP into a pHRSIN vector (generated by O. Lancho in a collaboration between our and M. Toribio's laboratories) were obtained by transient transfection of HEK-293T cells using Lipofectamine Reagent (Invitrogen). Three plasmids were transfected: HIV-derived psPAX2 (gag/pol) and pMD2G (VSV envelope), and the different lentiviral pHRSIN vectors. Culture supernatants were collected one and two days after transfection and ultra-centrifuged. The pellets containing the lentiviral particles were re-suspended in PBS ($1x10^8$ TU/ml). Preparation of lentiviral particles was performed by M.J. Martin-Bermejo. Small volumes of 2.5µl of GFP or SFRP1-IRES-GFP lentiviral particles were delivered into the lateral ventricle in the following stereotaxic coordinates of 0.5mm anterior/posterior, -1.0mm lateral, and -2.3mm dorsal/ventral from bregma, following the same procedure described above. One or five months post-injection, mice were sacrificed and processed for histological analysis.

3. Experimental Autoimmune Encephalomyelitis

Chronic EAE was inducted as described (Borroto et al., 2016). Briefly, female C57BL/6J and *Sfrp1*$^{-/-}$ littermates of 8 to 10 weeks old mice were subcutaneously injected with 150mg of $MOG_{35–55}$ (Espikem) emulsified in Freund's complete adjuvant (Sigma Aldrich) and supplemented with *Mycobacterium tuberculosis* (1mg/ml) (H37Ra strain from Difco) into both femoral regions. The mice were immediately injected intraperitoneally with 200ng of pertussis toxin (Sigma Aldrich) and, again, 48 hours after immunization. The animals were weighed and inspected for clinical signs of the disease on a daily basis by an observer blind to the genotype. Clinical signs of EAE was assessed according to a severity scale: 0) normal behaviour, no overt signs of disease; 1) weakness at the distal portion of the tail; 1.5) complete flaccidity of the tail; 2) moderate hind limb weakness; 2.5) severe hind limb weakness; 3) ataxia; 3.5) partial hind limb paralysis; 4) complete hind limb paralysis; 4.5) complete hind limb paralysis accompanied by muscle stiffness; 5) moribund state and hence sacrificed according to ethical procedures.

At day 16 after immunization, when the symptoms reached the peak of severity in the wild type, a representative pool of mice were anesthetized and perfused intracardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH7.6). The spinal

cords of the mice were dissected out and processed for histological analysis by immunostaining. Statistical analysis was performed using Prism software (GraphPad) by Mann–Whitney U nonparametric test.

4. Primary cultures

Glial primary cultures were established from cerebral cortices of newborns C57BL/6J or Sfrp1$^{-/-}$ mice no older than three days. Cortices were dissected in Ca^{2+} and Mg^{2+} free Hank's Balanced Salt Solution (HBSS from Invitrogen). Tissue was finely chopped and gently and mechanically dissociated with a Pasteur pipette, then incubated in HBSS for 10 minutes at 37ºC to allow auto-proteolysis in presence of 50µg/ml DNase1 (DN25 from Sigma Aldrich). After that, cells were centrifuged for 8 minutes at 1000rpm. The pellet was re-suspended in Dulbecco's modified Eagle medium and F-12 nutrient mixture (DMEM/F12 from Invitrogen) containing 10% Fetal Calf Serum (FCS from Invitrogen), and gentamycin (Sigma Aldrich). Approximately cortices from 2 pups were platted in 75cm^2 flask pre-treated with Poly-D-Lys (P7280 from Sigma Aldrich). Cells were cultured at 37ºC in a humidified 5% CO2 incubator. The day after, the medium was replaced by fresh one containing 10% conditioned medium from the L929 cell line containing m-CSF1 to improve microglial survival. The medium was not replaced for 2 weeks, when confluence was normally achieved. For the analysis of mixed cultures, cell cultures were detached as described (Saura et al., 2003). Briefly, cells were washed with warm phosphate buffer saline (PBS), and then incubated with 0.25% trypsin (Invitrogen), 1mM EDTA in PBS at 37ºC for 15min, until the majority of cells detached. After the addition of DMEM/F12 supplemented with 10% FCS, cells were centrifuged for 5 minutes at 1000rpm, and re-suspended in DMEM/F12 10% FCS.

Purified microglial cultures were obtained by mechanical detachment of mixed glial cultures. Flask containing mixed glial cultures were shaken and hand beaten for 15 minutes and then microglia was collected from the media. To maintain microglial proliferation, the flask was filled with 50% of new growth medium and 50% of the recovered medium. After 5 minutes centrifugation at 1000rpm cell pellet was re-suspended in DMEM/F12 10% FCS.

In both cases, cells were then seeded in different conditions depending on the experiment. For immunostaining, cells were platted on Poly-D-Lysine coated glass coverslips of 12mm diameter (Thermo scientific) at a density of 30.000 cells per cm^2. For protein or RNA quantification, cells were seeded directly on multi-well tissue culture plates (Falcon) at a density of 10^5 cells per cm^2. After 48 hours, cells were treated with different agents. Different stimuli were diluted in DMEM/F12 0,5% FCS. Cells were treated with *Escherichia coli* 0111:B4 lipopolysaccharide (LPS; L3024 from Sigma Aldrich) at 1µg/ml, interleukin 6 (IL6, provided from Dr. Alarcón, eBiosciences) at 10ng/ml, human recombinant SFRP1 protein (SRP3154 from Sigma Aldrich) at 0,5µg/ml, or Sfrp1 specific inhibitor (BML287 from Enzo Live Sciences) at 5µM. After treatment, cell media were collected and directly frozen at -80ºC. Cells for imaging were fixed with warm 4% paraformaldehyde (PFA30525 from Millipore) for 15 minutes at 37ºC and then washed twice with PBS. Covers were maintained at 4ºC in PBS 0.025% sodium azide until processed for immunocytochemistry. Cells for protein or RNA quantification were directly processed and stored at -80ºC.

5. qRT-PCR

After treatments, cultured cells were collected in cold PBS and total RNA was extracted with Trizol Reagent (Life technologies) following the protocol suggested by the manufacturer. RNA was purified with RNeasy Lipid Mini Kit (Qiagen) following the manufacturer's protocol. The concentration of the samples was determined at 260nm absorbance using a Nanodrop 1000 spectrophotometer (Thermo Scientific). Same amounts of RNA were retro-transcribed using the First-Strand cDNA Synthesis Kit (GE Healthcare) following the manufacturer's guidelines to obtain a concentration of 2.5µg/µl supposing a 100% effectivity in reaction. Quantitative PCR was accomplished following the protocol suggested by the manufacturer using Power SYBR Green Master Mix (Applied Biosystems) in reactions of 10µl and 10ng total cDNA mold. The primers were designed using Primer-Blast tool from NCBI. In all cases, the amplified region was around 250 base pairs and covering an exon-exon junction (probes are listed in Table1). GAPDH probe was used in parallel assay as housekeeping normalizer. The assay was performed at least in triplicate in 384-well optical plates using an ABI Prism 7900 Sequence Detection System (Applied Biosystem). All data were captured using the

Sequence Detector Software (SDS version 2.4, Applied Biosystems). Samples were analysed with the double delta CT (ΔΔCT) method, and fold change was calculated using the equation $2^{-\Delta\Delta CT}$. Statistically significant differences between samples were assessed by Student's t-test.

Probe	Forward primer	Reverse primer
GAPDH	AAAATGGTGAAGGTCGGTGTGA	ATGGGCTTCCCGTTGATGAC
Arg1	ACATCAACACTCCCCTGACAAC	GCCAATCCCCAGCTTGTCT
IL1β	GCACTACAGGCTCCGAGATG	TGGGTGTGCCGTCTTTCATT
IL6	CACTTCACAAGTCGGAGGCTT	GGTACTCCAGAAGACCAGAGGA
IL10	AGTGGAGCAGGTGAAGAGTGA	TCGGAGAGAGGTACAAACGAGG

Table1: List of qRT-PCR primers used in this Thesis.

6. Immunohistochemistry

Newborn and adult mice were transcardially perfused with 4% PFA in phosphate buffer 0,1M (wt/vol). Brains were removed, post-fixed by immersion overnight and then washed for one day in PBS, incubations were made in a rocking platform at 4ºC. Spinal cords was extracted after whole body post-fixation and washing to avoid tissue collapse. After washes, tissues were incubated in a 30% sucrose-PB solution (wt/vol) during 24 hours for cryo-protection. Tissue was embedded and frozen on dry ice in a 7.5% gelatin in 15% sucrose solution (wt/vol). Frozen tissues were stored at -80ºC until serially sectioned in the coronal plane at 15µm of thickness using a cryostat (Leica).

Both histological and cytological immunostaining were performed following standard protocols. After defreeze, sections were washed three times with PBS for 10 minutes. For certain primary antibodies, such as AbCAM Sfrp1 and Aβ, antigen retrieval was necessary. Heat treatment was performed in citrate buffer 10mM at pH6, boiling the sections at 110ºC for 5 minutes under controlled pressure conditions (BIOCAREMEDICAL). After that, the endogenous peroxidase was blocked with methanol 3% H2O2 for 10 minutes. Three washes for 5 minutes were performed with PBS 0,1% Triton X-100 (PBT). For CD11b immunostaining detergent was omitted. Sections were blocked with blocking buffer (PBT 5% FBS 0,1% BSA) at least for one hour at room temperature. Subsequently, sections were incubated with primary antibodies (resumed in Table2) diluted in blocking buffer overnight at room temperature, in cytological immunostaining incubation was performed at 4ºC in a rocking platform.

Antibody Anti-	Source	Host	Dilution
Aβ (4G8)	Covance	Mouse monoclonal	1:500
Aβ (6E10)	Covance	Mouse monoclonal	1:500
β-galactosidase	AbCAM	Chicken polyclonal	1:2000
CD11b	BD	Rat monoclonal (biotin)	1:200
CD4	BD	Rat monoclonal (biotin)	1:200
CD45	Serotec	Rat monoclonal	1:500
GFAP	Dako	Rabbit polyclonal	1:1000 / 1:3000
GFAP (GA5)	Millipore	Mouse monoclonal	1:1000 / 1:2000
GFP	AbCAM	Chicken polyclonal	1:2000
Iba1	Wako	Rabbit polyclonal	1:1000 / 1:3000
Iba1	Millipore	Mouse monoclonal	1:1000 / 1:2000
MBP	AbCAM	Rabbit polyclonal	1:1000
NeuN	Millipore	Mouse monoclonal	1:1000
SFRP1	AbCAM	Rabbit polyclonal	1:1000 / 1:3000
SFRP1	Sigma	Rabbit polyclonal	1:1000 / 1:3000
SFRP1	Cell Signaling	Rabbit polyclonal	1:1000
SFRP1	*M. Dominguez**	Mouse monoclonal	1:1000 / 1:2000
TauP (AT8)	Thermo	Mouse monoclonal	1:50

Table2: List of primary antibodies used for immunostaining in this Thesis. Source, type, and dilution are indicated. Dilution antibodies used in tissue/cells. *Antibody generated by our laboratory in collaboration with the laboratory of M. Dominguez (ISCIII).

After incubation with primary antibody, three washes for 10 minutes were performed with PBT. Secondary antibodies were used at the indicated dilutions and incubated for one hour at room temperature: donkey anti-rabbit Alexa488, donkey anti-rabbit Alexa594, donkey anti-mouse Alexa488, donkey anti-mouse Alexa594, goat anti-chicken Alexa488, goat anti-rat Alexa594, 1:1000/1:3000 (Molecular Probes, Invitrogen); Streptavidin Alexa488, Streptavidin Alexa594, 1:500 (Molecular Probes, Invitrogen); goat anti-mouse biotin, goat anti-rabbit biotin, 1:500 (Jackson Lab). In some cases, biotin conjugated antibodies were used in combination with Streptavidin-POD (Jackson Lab). Antibody staining was revealed with Diaminobenzidine. Sections were counterstained with Hoechst (Sigma Aldrich). For fibrillary plaques detection sections were stained with Thiofalvin S in some cases. All samples were mounted with Mowiol (Calbiochem).

The preparations were analysed with a microscope DMCTR5000 equipped with a DFC350Fx monochrome camera or a DFC500 colour camera (Leica Microsystems). Confocal imaging was realised with a confocal laser scanning microscope LSM710 coupled to a vertical microscope AxioImager.M2 (Zeiss). Fluorescence quantification was assessed with ImageJ software (National Institute of Health). 3D reconstruction and volumetric quantitation was performed using Imaris software (version 7.1.1, Bitplane).

7. Western Blot and Immunoprecipitation

To obtain cellular protein fraction, tissue or cultured cells were equally treated with RIPA buffer (NP-40 1%, Sodium deoxycholate 0.5%, SDS 0.1% in PBS 1X) complemented with Proteases Inhibitor Complex (Roche) and PMSF. Tissue was homogenised and disaggregated, previous to disrupt by sonication. Homogenate was stabilised on ice for 15 minutes and next centrifuged at 15000rpm for 30 minutes at 4ºC. Supernatants were collected and protein concentration was quantified by BCA protein assay kit (Thermo Scientific). Different samples were diluted at the same concentration and subsequently diluted in Laemlli Buffer 5X (Glycerin 50%, SDS 10%, 0,025% Bromophenol blue, 10% β-mercaptoethanol in 20mM TrisHCl pH6.8). Cell supernatants were directly diluted in Laemlli Buffer. After re-suspension lysates were denaturalised at 100ºC for 5 minutes.

In the case of Immunoprecipitation, cells were collected in cold PBS and treated with Brij96 lysis buffer (Brij96 0.33% v/v, NaCl 150mM in 20mM TrisHCl pH7.8) complemented with Protease Inhibitor Complex (Roche) and PMSF 1mM. The cellular homogenate was stabilised for 45 minutes on ice and next centrifuged at 15000rpm for 15 minutes. Supernatant aliquot was separated as Input and the rest was incubated for 4 hours with three subsequent preclearing protein G-agarose beads (Sigma Aldrich), previously incubated overnight with an unspecific antibody of the same isotype as desired (Mouse IgG1 Isotype Control, R&D Systems). After preclearing, the samples were centrifuged at 3000rpm for 3 minutes at 4ºC, and the supernatant was next incubated for 4 hours with protein G-agarose beads previously incubated overnight with target antibody (2µg of antibody per 40µl beads). Washes of the beads were performed before and after incubation with the lysate to avoid unspecificities. Beads were finally re-suspended in Laemlli Buffer 2X and denaturalised at 100ºC for 5 minutes.

All samples were resolved in SDS-PAGE gels of acrylamide/bis-acrylamide 8-12% and analysed by Western Blot. Proteins were transferred to nitrocellulose membranes by dry iBlot2 (Invitrogen) and incubated with TBS (NaCl 150mM in TrisHCl 10mM pH8) containing 0.1% Tween (TBST) and 10% non-fat milk for at least one hour. After blocking, the membranes were incubated overnight at 4ºC with primary antibodies (described in Table3) diluted in 5% Bovine Serum Albumin (Sigma Aldrich) in TBST. Afterwards, the membranes were washed three times for 10 minutes with TBST, and incubated for one

hour at room temperature with secondary antibodies linked to POD (Jackson Lab) diluted in 10% non-fat milk in TBST. Membranes were then washed twice with TBST and once again with TBS before visualization with ECL Advanced Western Blotting Detection Kit (Amersham). Signal intensity was quantified by densitometry using Image Lab Software (version 5.2.1, BioRad).

Antigen	Source	Dilution
Aβ (6E10)	Covance	1:1000
N-Cadherin	Invitrogen	1:500
IL1β	R&D Systems	1:5000
SFRP1	Cell Signalling	1:1000
αTub (B512)	Sigma Aldrich	1:3000
GAPDH	Thermo Fisher	1:1000
FLAG	Hybridoma Bank	1:1000
HA (12CA5)	Hybridoma Bank	1:1000
c-myc (9E10)	BD Pharmingen	1:1000

Table3: List of primary antibodies used for Western Blot analysis.

8. Analysis of ADAM10 activity

In order to determine the activity of ADAM metalloproteases, we used a Fluorogenic Peptide Substrate (R&D Systems) with the following sequence Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2. The peptide fluorescence only when is cleaved by ADAM8, ADAM9, ADAM10 or ADAM17. The peptide contains a highly fluorescent 7-methoxycoumarin group that is efficiently quenched by the 2,4-dinitrophenyl group. When ADAM cleave the amide bond between the fluorescent group and the quencher group, fluorescence is activated. 10^5 microglial cells/well were platted into a BioLite 96 well Multidish (Thermo Scientific) and stabilised in DMEM/F12 0.5% FCS. The day after, microglial cells were incubated in darkness at 37ºC for 4 hours with the fluorogenic peptide at a final concentration of 6µM diluted in HBSS (Invitrogen). In the presence or absence of 0,5µg/ml human recombinant SFRP1 (Sigma) or 5µM ADAM10 specific inhibitor GI254023X (R&D Systems). The fluorescence was measured in a fluorescence plate reader FLUOstar OPTIMA (BMG LABTECH) with excitation at 320nm and emission at 405nm.

For the analysis of hTREM2 shedding we used a stably transfected HEK-293 FLP-in cell line, expressing a double-tagged hTREM2 with an HA tag at the N-terminal and a FLAG tag at the C-terminal. A control line, carrying the empty pCDNA5 was used as control

(kindly provided by G. Kleinberger in a collaboration between our and C. Haass' laboratories).

To analyse the ADAM10 shedding activity of CD200 and CX3CL1, we prepared two expression constructs in the pCMV3 plasmid carrying the entire coding sequence of each murine gene (RefSeq NM_010818.3 and NM_009142.3 respectively) double-tagged with FLAG tag in the N-terminal and HA tag in the C-terminal. To obtain these constructs we modified the CD200 MG50074-CY and MG50074-NF, and the CX3CL1 MG50917-CY and MG50917-NF constructs from SinoBiological. These plasmids were co-transfected with pCAG-GFP 5:1 into HEK-293T cell line with Lipofectamine Plus Reagent (Invitrogen). Three days after, transfection efficiency was assessed by GFP fluorescence under a fluorescence scope M205FA (Leica).

Cells expressing either hTREM2, CD200 or CX3CL1 were treated with 5µM ADAM10 specific inhibitor GI254023X (Sigma), 25µM of ADAM broad inhibitor GM6001 (kindly provided by M.J. Bullido, Enzo Live Sciences), or 0,5µg/ml human recombinant SFRP1 (R&D Systems). One-day after treatment, cell culture supernatants were collected and analysed by Western Blot as described.

9. ELISA

Quantitative determination of cytokine content in cell culture supernatant was assessed by electro-chemo-luminescence in MSD MULTI-SPOT Assay System in 96 well V-PLEX plates of Pro-Inflammatory Mouse Panel 1 or custom Mouse Cytokine V-PLEX plates for IFNγ, IL1β, IL4, IL6, IL10 and TNFα (Meso Scale Discovery). The assay was performed following the manufacturer's indications and measured on a SECTOR Imager 2400 reader (Meso Scale Discovery).

Quantitative determination of SFRP1 protein levels in cell culture supernatant or RIPA fraction of brain lysates were assessed by a capture ELISA developed in our laboratory. 96-well microtiter plates (Nunc) were coated overnight at 4ºC with 50µl/well of 1.5µg/ml anti-SFRP1 in PBS. Plates were washed 3 times with 0,05% Tween20 in PBS (PBST) and incubated for three hours with 2% BSA in PBST at room temperature. Plates were washed again with PBST, and incubated 2 hours at 37ºC with 50µl/well of samples. Culture supernatants were diluted five folds, and brain lysates were incubated at protein

concentration of 0.1µg/µl, previously quantified by BCA protein assay kit (Thermo Scientific). Wells were washed with PBST and incubated for one hour at 37ºC with 50µl/well of biotin-labelled anti-SFRP1 at 1µg/ml. Plates were further washed with PBST and incubated for one hour at room temperature with 50µl/well of Streptavidin-POD 1:2000 (Jackson Lab). After extensive washing with PBST, the enzymatic reaction was developed at room temperature in the dark for about 20 min using 100µl/well of Tetra-methyl-benzidine liquid substrate (TMB, Sigma Aldrich). The reaction was terminated by the addition of 100µl/well of HCl 2N and the resulting product was measured at 450nm in a microtiter plate ELISA reader FLUOstar OPTIMA (BMG LABTECH).

RESULTS

1. Sfrp1 acts as a pro-inflammatory molecule *in vitro*

Sfrp1 is expressed by glial cells

To determine whether Sfrp1 might be involved in the regulation of neuroinflammation, we first sought to determine its expression pattern. Sfrp1 is abundantly expressed in the developing CNS, including the eye and the telencephalon (Esteve et al., Submitted; Esteve et al., 2011a; Kim et al., 2001), but its expression is down regulated in the adult brain (Augustine et al., 2001), with moderated expression restricted to the cerebellum, olfactory bulbs, sub-ventricular zones and the rostral migratory stream. Notably, Sfrp1 appeared to be expressed by glial cell in the adult brain (GENSAT and Fig.5). To determine if glial cells were indeed a possible source of Sfrp1 we first established primary cortical cultures from neonatal brains. These cultures were analysed by co-immunostaining with antibodies against Sfrp1 and proteins that serves as reliable specific markers for microglia, astrocytes and neurons. As shown in Fig.6, Sfrp1 is strongly expressed by microglial cells and astrocytes, although in the latter only at lower levels. No expression was observed in neurons.

This expression confirms that glial cells are among the possible sources of Sfrp1 in the brain.

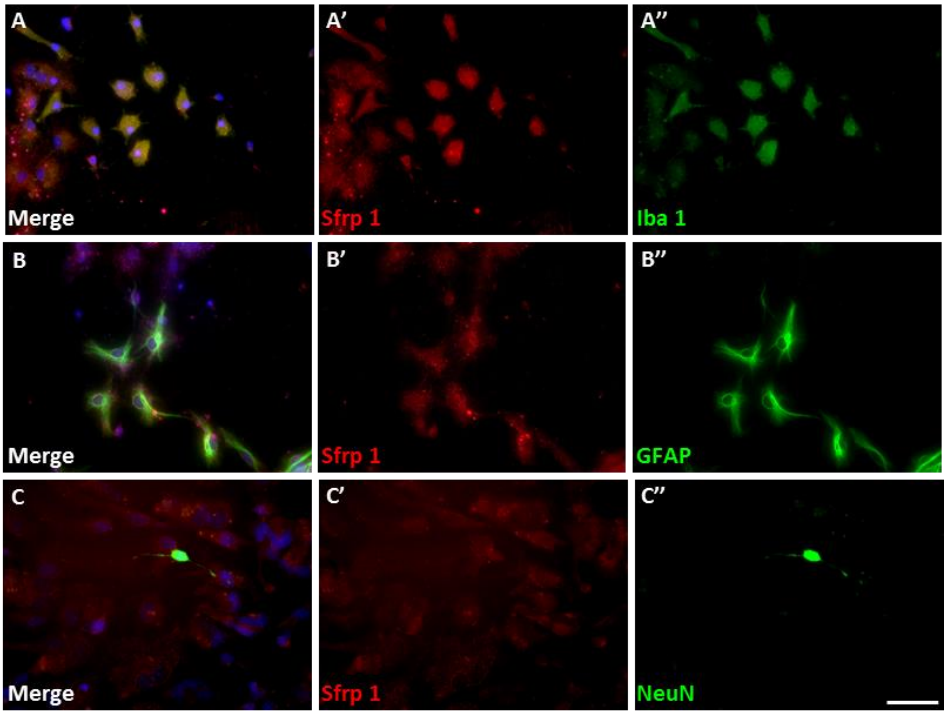
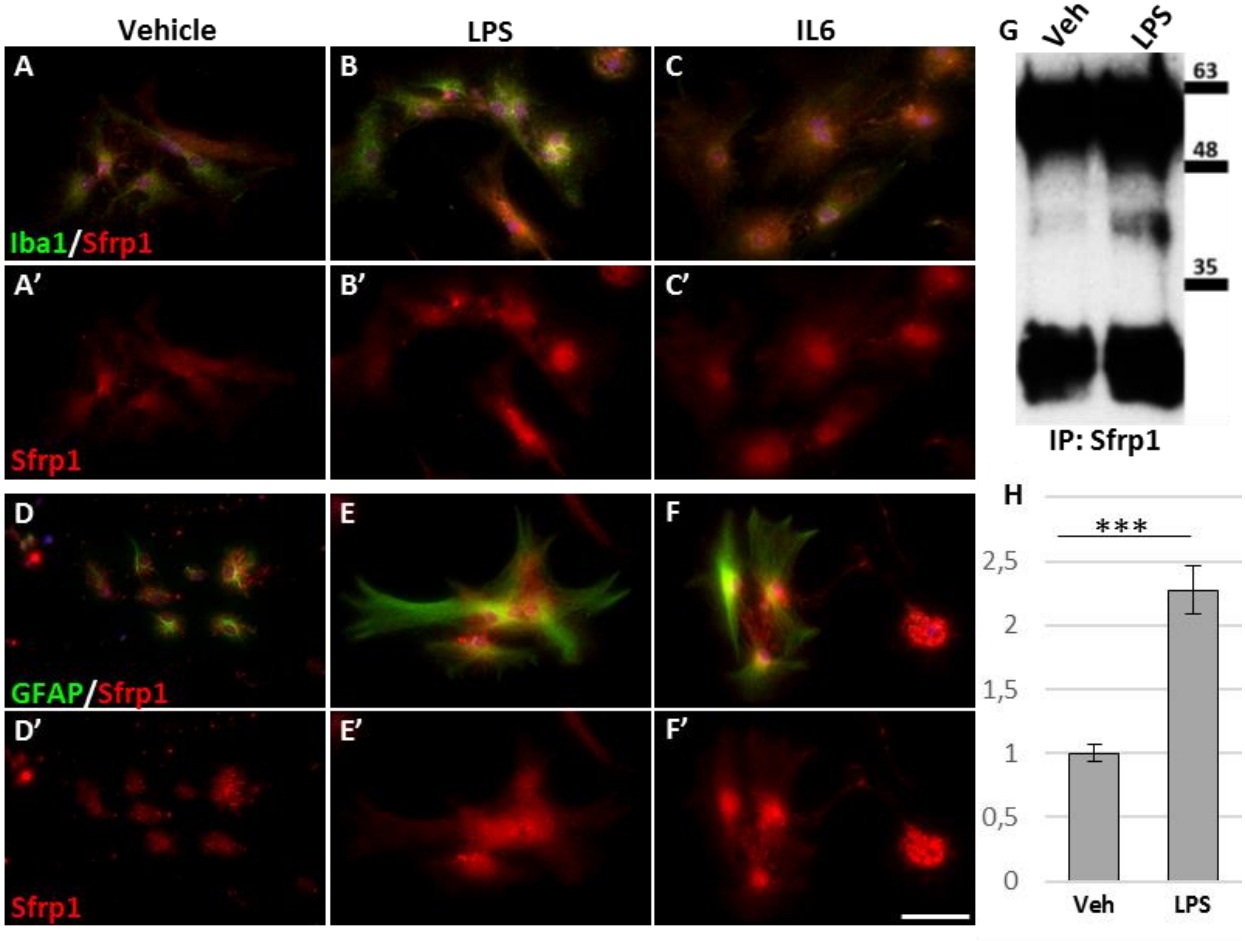


FIG.6: Sfrp1 is expressed by glial cells *in vitro*. Mixed cultures from neonatal cortex were co-immunostained with antibodies against Sfrp1 (in red) and microglial (Iba1 in green, A), astrocytic (GFAP in green, B) or neuronal markers (NeuN in green, C). Note Sfrp1 expression in microglial cells and astrocytes but not in neurons. Scale bar 60µm.

Pro-inflammatory stimuli induce Sfrp1 expression

We next sought to determine if the expression of Sfrp1 changes in response to an inflammatory stimulus. Mixed glial cultures were treated for 24 hours with LPS (1µg/mL) or Interleukin 6 (IL6; 10ng/mL), two commonly used pro-inflammatory stimuli. LPS, a lipopolysaccharide found in the outer membrane of Gram-negative bacteria, acts as an endotoxin and elicit a strong immune response (S. D. Wright, 1999). IL6 is a potent mediator of the acute phase of inflammation (Sochocka et al., 2016).

In response to both pro-inflammatory stimuli, Sfrp1 expression underwent a notable increase in both activated Iba1-positive microglia and GFAP-positive reactive astrocytes (Fig.7), although this increase was more prominent in microglial cells. As expected, after an inflammatory challenge, cells showed also the typical morphological changes of reactive astrocytes with the presence of larger and thicker processes (Fig.7D-F). Microglial cells instead adopted an amoeboid shape, as revealed by Iba1 immunohistochemical staining (Fig.7A-C).

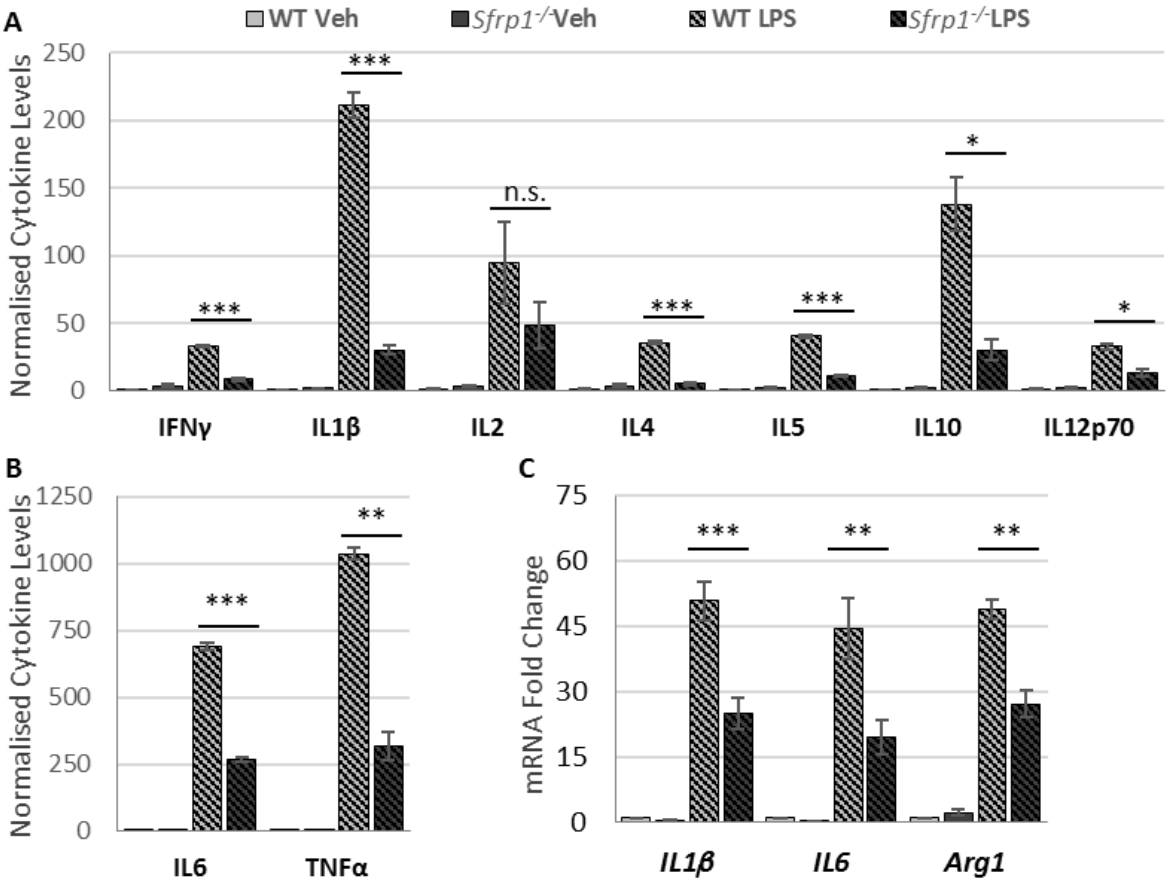


Sfrp1 increase was verified by immunoprecipitation and Western blot comparison of the protein content in the culture media of LPS-treated and untreated cultures (Fig.7G), consistent with the highly secreted nature of Sfrp1 protein. Similar results were obtained using a homemade ELISA (Fig.7H), which revealed that Sfrp1 content in the media of LPS-treated cells was more than double of that of control cultures.

Lack of Sfrp1 reduces cytokine production and secretion

CNS inflammation is inevitably linked to the activation of glial cells. When activated, glial cells acquire a polarized phenotype characterized by a different morphology and different functions required to respond to the insult the CNS has suffered. The main outcome of this activation is the ability to secrete and respond to a large number of cytokines (Shichita et al., 2014). The differential activation of glial cells triggers distinct profiles of cytokine secretion to promote distinct outputs, conferring glial cells with a pivotal role in modulating the type and extent of the neuroinflammatory response (Heneka et al., 2014). Glial polarization has been widely studied in the case of microglial cells and there are a number of well-described activation markers that are typically used to characterize the so-called M1 or M2 state of microglial cells. The M1 phenotype is usually defined by the expression of pro-inflammatory cytokines, whereas the M2 state by the production of anti-inflammatory cytokines (Colton, 2009).

FIG.7: Sfrp1 expression increases in response to pro-inflammatory stimuli. New-born mixed glial cultures (A-F) treated with vehicle (A,D), 1µg/ml LPS (B,E) or 10ng/ml IL6 (C,F) for one day were co-immunostained with antibodies against Sfrp1 and a microglial (Iba1; A-C) or astrocytic markers (GFAP; D-F). Sfrp1 staining (red) increases upon LPS and IL6 treatment, compared to vehicle treated cultures. Note that pro-inflammatory stimuli induce morphological changes in activated microglia (amoeboid; B,C) and reactive astrocytes (larger processes; E,F). G) Immunoprecipitation and Western Blot analysis of the levels of Sfrp1 protein in vehicle or LPS treated cultures revealed that Sfrp1 is enriched in the medium of the cultures treated with LPS. H) ELISA quantification of Sfrp1 levels present in culture supernatants show a 2.25 fold increase of Sfrp1 in response to LPS. Error bars represent Standard Error. Statistical significance: ***P<0.001 by Student's t-test. Scale bar 60µm.



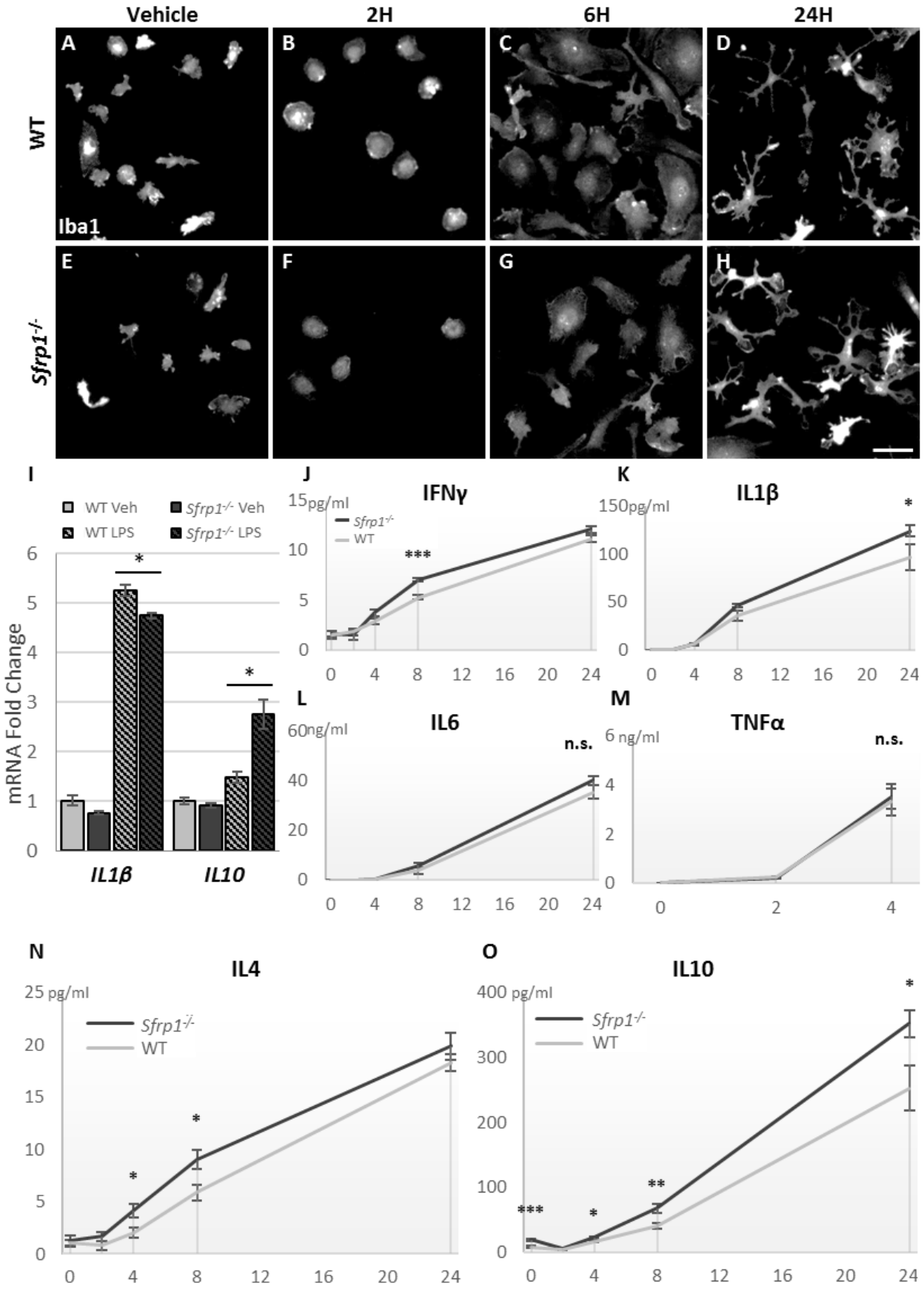
We determined the expression of M1/M2 specific polarization markers in primary glial cultures from *Sfrp1* null and wild type mice treated for one day with or without LPS (1µg/ml) (Fig.8). Q-RT-PCR analysis showed that, upon stimulation, the mRNA both M1 (IL1β and IL6) and M2 (Arg1) markers was poorly expressed in *Sfrp1* null cells as compared to the wild type ones (Fig.8C). In parallel, we determined the secretion profile of anti- and pro-inflammatory cytokines in the culture media of the treated and untreated cultures using an electroluminescence ELISA from MesoScale (Fig.8A,B). In the culture media from *Sfrp1* null glia, there was a significant reduction of the amount of all the tested cytokines, independently of their pro- (IL1β,IL6,TNFα,IFNγ,IL12) or anti-inflammatory (IL4,IL10) activity. Notably, the highest differences were observed for those cytokines also produced by astrocytes (IL1β,IL6,IL10,TNFα) (Qin & Benveniste, 2012).

FIG.8: Glial derived cytokine secretion is reduced in the absence of Sfrp1. Primary glial cultures from wild type (WT) and *Sfrp1* null mice were analysed after 24 hours of continued exposure to 1µg/ml LPS or vehicle (Veh). Cytokine content in cultures supernatants was determined by ELISA. Values were normalised to those of the untreated wild type cultures (A,B). Note the lower levels of cytokine secretion in response to LPS in the cultures from *Sfrp1* null newborn mice for all the cytokines tested. qPCR analysis of IL1β and IL6 markers (C) of M1, and Arg1 of M2 phenotype. Data are represented as Fold Change (calculated by $2^{-\Delta\Delta Ct}$) normalized to those of the untreated wild type. Lower mRNA levels for all analysed markers were observed in cultures from *Sfrp1* null mice compared to wild type after LPS treatment. Error bars represent Standard Error. Statistical significance: *P<0.05, **P<0.01, ***P<0.001 by Student's t-test comparing between LPS treated.

Microglial activation is reduced in the absence of Sfrp1

The consistent reduction of the expression levels of all the analysed cytokines in the *Sfrp1*$^{-/-}$ culture suggests that normally Sfrp1 promotes the activation of glial cells, whereas its absence favours their resting state.

It has been proposed that activation of astrocytes is secondary to that of microglial cells (Holm et al., 2012). We therefore hypothesized that microglia could be the main target of Sfrp1 activity. To test this possibility, we established pure primary microglial cultures and observed their morphological changes during treatment with LPS (1µg/ml) (Fig.9A-H). Soon after exposure to LPS, microglial cells transform from cells with a small soma and little filopodia into cells with an amoeboid shape and large lamellipodia that increase in length with time, assuming the morphology of a typical migrating cell (Fig.9C). This is considered as a transformation towards a macrophagic phenotype, often observed after brain injury, which likely enables microglial cell to produce inflammatory cytokines and to engage in phagocytosis (Ransohoff & Perry, 2009). After this initial reaction, microglial cells undergo a secondary morphological change, acquiring a characteristic ramified shape with multiple lamellipodia (Fig.9D), and a preponderant phagocytic profile, known as the alternative state of activation (Perry et al., 2007). Notably, all these changes were rather delayed in microglial cells cultured from *Sfrp1* null brains (Fig.9E-H). In the presence of LPS, *Sfrp1*$^{-/-}$ microglial cells presented a slower growth of the lamellipodia and a smaller size as compared to that of the wild type at the same time points (in Fig.9, compare F,G with B,C). Nevertheless, the morphological changes of the *Sfrp1* null microglia were less evident and delayed but they still took place so that at the end of the analysis, there was no appreciable difference between wild type and *Sfrp1* null microglia (Fig.9D,H).


Vehicle
2H
6H
24H
A
B
C
D
WT
Iba1
E
F
G
H
Sfrp1-/-
I
WT Veh
Sfrp1-/- Veh
WT LPS
Sfrp1-/- LPS
mRNA Fold Change
IL1β
IL10
J
IFNγ
15pg/ml
Sfrp1-/-
WT
***
K
IL1β
150pg/ml
*
L
IL6
60ng/ml
n.s.
M
TNFα
6 ng/ml
n.s.
N
IL4
25 pg/ml
Sfrp1-/-
WT
O
IL10
400 pg/ml
Sfrp1-/-
WT

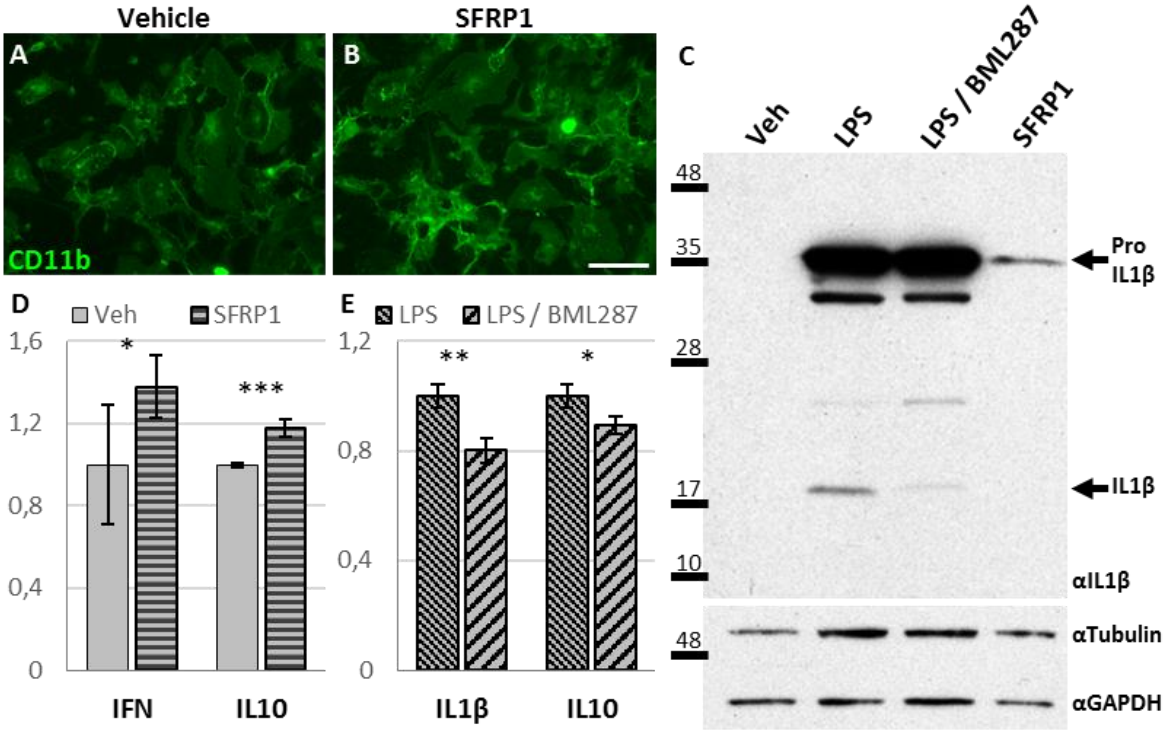
To confirm these observations, we analysed the cytokine secretory profile of *Sfrp1*$^{-/-}$ and wild type microglial cells in response to LPS (Fig.9J-O). In contrast with the results obtained with mixed microglia-astrocytes cultures, we did not observe changes in the levels of IL1β, IL6 and TNFa release in the absence of Sfrp1 (Fig.9K-M). Instead, IFNγ secretion was significantly increased in *Sfrp1* null microglia 8 hours after LPS treatment (Fig.9J). When anti-inflammatory cytokines were analysed, we determined that *Sfrp1* null microglia secrete significantly more IL4 at 4-8 hours after LPS treatment (Fig.9N) There was also a consistent increase of IL10 release to the media of *Sfrp1* null microglial cultures and, interestingly, even the basal level of IL10 secretion was higher in null microglia than in wild type (Fig.9O). To analyse further the differential behaviour of *Sfrp1* null microglia to LPS stimulation, we determined their early response by measuring the mRNA levels of one pro- and one anti-inflammatory cytokine after 4 hours of exposure to LPS (Fig.9I). qPCR analysis of the pro-inflammatory cytokine IL1β showed a mild decrease of expression in *Sfrp1* null microglia as compared to wild type. In contrast, the mRNA levels of the anti-inflammatory cytokine IL10 was significantly increased in *Sfrp1* null microglia as compared to wild type.

In sum, *Sfrp1* null microglial cultures respond to LPS with an increased secretion of anti-inflammatory cytokines but little modification of the secretory pro-inflammatory cytokine profile, suggesting that Sfrp1 likely makes microglial cells more prone to polarize towards a pro-inflammatory state whereas in its absence microglial cells seem to be polarized towards a M2 state.

FIG.9: The absence of Sfrp1 polarizes microglial cells towards an anti-inflammatory state. Purified microglial cultures from wild type (WT) and *Sfrp1*$^{-/-}$ mice were treated with 1µg/ml LPS or vehicle for 24 hours. Morphological changes (A-H) and the profile of cytokine secretion (J-O) were analysed in a time course assay. mRNA levels of IL1β and IL10 were quantified by Q-RT-PCR at 4 hours post treatment with LPS or vehicle (I). Data are represented as Fold Change (calculated by $2^{-\Delta\Delta Ct}$) normalized to the untreated wild type. *Sfrp1* null microglia early response is characterised by lower expression of pro-inflammatory markers (IL1β) and higher expression of anti-inflammatory markers (IL10). Cultures were fixed at initial time point (A,E), 2 (B,F), 4 (C,G) and 24 (D,H) hours post LPS treatmet, and microglial cells were stained with an antibody against Iba1. A delay in morphological changes could be observed, such as reduced lamellipodia, in the *Sfrp1* null microglia compared to the control. Wild type and *Sfrp1* null microglial cultures treated with LPS were quantitatively analysed by ELISA for diverse cytokine secretion in a time course assay: IFNγ (J), IL1β (K), IL6 (L), TNFα (M), IL4 (N) and IL10 (O). Note the mild changes in pro-inflammatory cytokines (J-M) in contrast to the consistent changes observed in anti-inflammatory cytokines (N,O). Error bars represent Standard Error. Statistical significance: n.s. P>0.05, *P<0.05, **P<0.01, ***P<0.001 by Student's t-test comparing between LPS treated (I) and genotypes at the same time point (J-O). Scale bar 60µm.

Sfrp1 addition is sufficient to activate microglial cells

To test the above hypothesis we asked if the addition of Sfrp1 was sufficient to induce an inflammatory response in glial cells (Fig.10). Primary microglial cultures treated with 500ng/ml of human recombinant SFRP1 for 24 hours appeared to activate and secrete significantly higher levels of cytokines (Fig.10D). The activation state of glial cells was determined by immunostaining with antibodies against CD11b, a well-described marker for the activated state of microglia (Fig.10A,B). This marker was highly expressed by microglial cells after SFRP1 treatment (Fig.10B). Furthermore, the addition of SFRP1 to purified microglial cultures induced the expression of Pro-IL1β as determined by Western Blot analysis of IL1β content in the culture lysate (Fig.10C). We next reasoned that if Sfrp1 was directly involved in the response of glial cultures to LPS, the addition of a Sfrp1 specific inhibitor (BML287) should interfere with it. The co-treatment of primary microglial cultures with LPS and BML287 caused a reduction of both IL1β and IL10 release in the culture media (Fig.10E). Furthermore, the analysis of IL1β content in microglial lysates revealed no differences in the expression of Pro-IL1β in the cultures co-treated with LPS and BML287 or with LPS alone, although the production of mature and active IL1β was apparently reduced when Sfrp1 activity was inhibited (Fig.10C).



2. Sfrp1 is necessary and sufficient to induce an inflammatory response *in vivo*

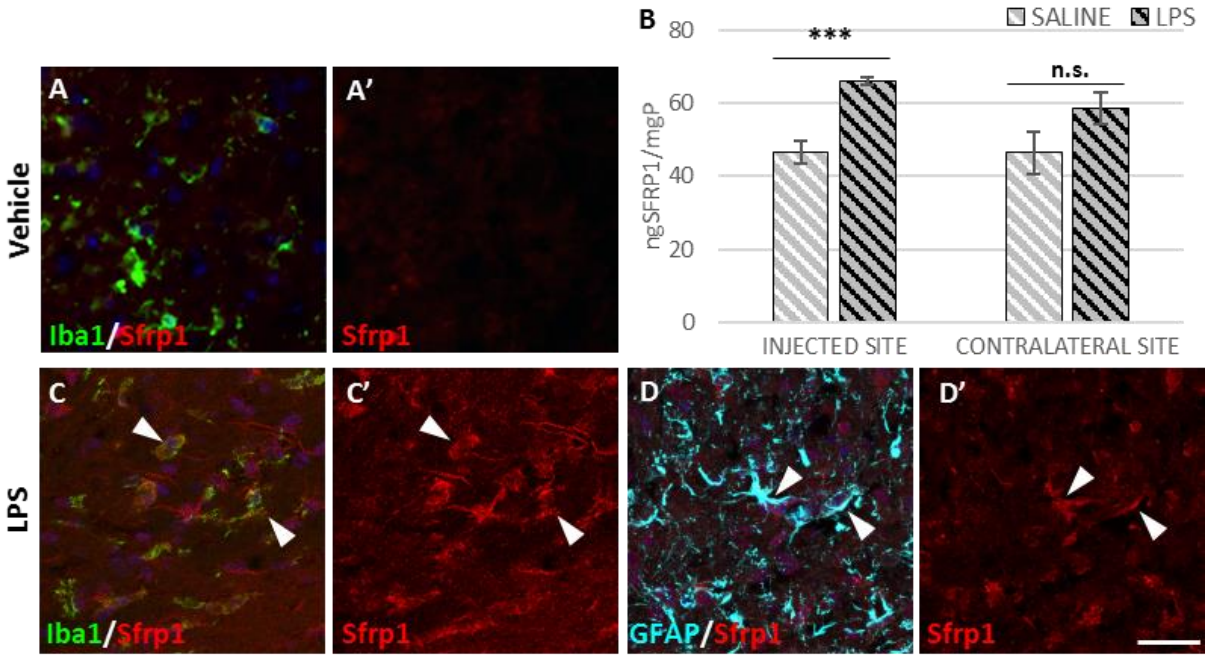
Altogether the results obtained with glial cell cultures provided evidence that Sfrp1 is sufficient to trigger microglial activation. However, under *in vitro* conditions, microglial cells behaviour differ from brain microglia, because the environment influences macrophage functions and gene expression (Gosselin et al., 2014). The next obvious question was if this occured also *in vivo*, where microglial are embedded into a different milieu, interacting with other cells types that also respond to insults.

To answer this question we took advantage again of LPS, as its infusion in the brain represents a broadly applied tool to induce brain inflammatory processes (S. D. Wright, 1999). Specifically, we infused LPS (5µg in total) directly into the brain parenchyma by intra-cerebral injection to groups of 4-6 animals, while controls were injected with saline. Three days after surgery, when the inflammatory process reaches its peak of activation (Rivest, 2009), the animals were sacrificed and the brains were analysed either by histological or biochemical procedures. The correct localization of the injection was verified during histological analysis, and badly injected animals were discarded.

FIG.10: SFRP1 treatment is sufficient to trigger an inflammatory response in cultured glial cells. A,B) Mixed glial cultures from wild type cortex treated with 500ng/ml human recombinant SFRP1 for 24 hours were stained with an antibody against CD11b (A,B). Note the higher levels of CD11b expression in the presence of SFRP1, indicating an activated state of microglial cells. C) Purified microglial cultures were treated with 500ng/ml SFRP1, 1µg/ml LPS, 5µM BML287, a specific Sfrp1 inhibitor, and/or vehicle for 24 hours. After treatment, cell lysates were assessed by Western Blot with an antibody against IL1β. Note that LPS induces a huge increment of Pro-IL1β (35KDa) expression and its cleavage into the active IL1β (17KDa) compared to vehicle treatment. Observe the reduced LPS mediated formation of mature IL1β in the presence of BML287. Conversely, SFRP1 addition induces the expression of Pro-IL1β. D,E) ELISA of cell supernatants to determine the content of IL1β and IL10 released by microglial cells. SFRP1 induces a significant increase in the secretion of both cytokines (D). BML287 prevents LPS-mediated release of both IL1β and IL10 (E). Error bars represent Standard Error. Statistical significance: *P<0.05, **P<0.01, ***P<0.001 by Student's t-test comparing between closed. Scale bar 60µm.

LPS induces Sfrp1 expression in vivo

We initially asked if LPS infusion was sufficient to upregulate Sfrp1 expression in wild type animals (Fig.11). After saline infusion, the brain of wild type animals presented no activation of glial cells (Fig.11A and Fig.12A), with the exception of a mild gliosis around the needle track. In contrast, the cortex of the animals infused with LPS presented a massive activation of microglial cells and astrocytes (Fig.11C,D and Fig.12B). Accompanying this activation, we observed an evident upregulation of Sfrp1 expression (in Fig.11, compare A' with C',D'). Colocalization studies with confocal microscopy indicated that Sfrp1 was localized in both microglia (Fig.11C) and astrocytes (Fig.11D), marked by anti-Iba1 and GFAP antibodies, respectively. To estimate the levels of Sfrp1 upregulation we used an ELISA approach (Fig.11B). Cubes of approximately 10mm^3 of the cortex at the injection site were dissected and lysated. Comparison of Sfrp1 levels from LPS treated and control tissue extracts showed an upregulation of about 40 %. A similar analysis of using the contralateral site revealed no statistically significant difference between control and LPS treated tissue. The mild variation may represent some linkage of LPS itself or of immunomodulators into the cerebrospinal fluid.



Sfrp1 ***null mice present a poor response to LPS***

We next determined if Sfrp1 is necessary to mediate an appropriate inflammatory response. To this end, we compare the effect of intra-cortical infusion of LPS into the brains of wild type and *Sfrp1* null mice (Fig.12). Brain sections from the experimental and control animals were immunostained with antibodies against GFAP and CD45 to determine the activation of astrocytes and microglial cells, respectively. As expected LPS, but not saline, treatment caused the appearance of strongly GFAP-positive astrocytes (in Fig.12 compare A,D with B,E) and CD45-positive microglial cells (in Fig.12 compare G with H) even further away from the injection site. In contrast, *Sfrp1* null littermates presented fewer and less immune-positive astrocytes (in Fig.12 compare B,E with C,F). Even more remarkable, null animals presented the near absence of CD45 positive activated microglia (in Fig.12 compare H with I). Quantitative comparison of the GFAP immunoreactivity among control and LPS treated wild type and *Sfrp1* null mice confirmed a significant difference in GFAP expression (Fig.12J). In wild type animals LPS induces a 90% increase in GFAP immunoreactivity with respect to saline treated animals. This increase barely reached 50% in *Sfrp1* null animals as, indicating a less reactive state. No statistically significant differences were observed when we compared wild type and *Sfrp1* null mice infused with saline.

FIG.11: Infusion of LPS in the adult mouse cortex induces Sfrp1 expression. Confocal microscopy analysis of cryostat sections from adult mouse brains three days after intra-cortical infusion of saline (A) or LPS (C,D). Sections were immunostained with antibodies against Sfrp1, Iba1 (A,C) and GFAP (D). Note the high expression of Sfrp1 in LPS infused brain. Sfrp1 colocalizes with microglial cells (arrowheads in C) and astrocytes (arrowheads in D) after LPS treatment. Levels of Sfrp1 expression were determined by ELISA (B) using extracts of cortical cubes of $10mm^3$ adjacent to the injection site. Error bars represent Standard Error. Statistical significance: n.s. P>0.05, ***P<0.001 by Student's t-test comparing between closed. Scale bar 25µm.

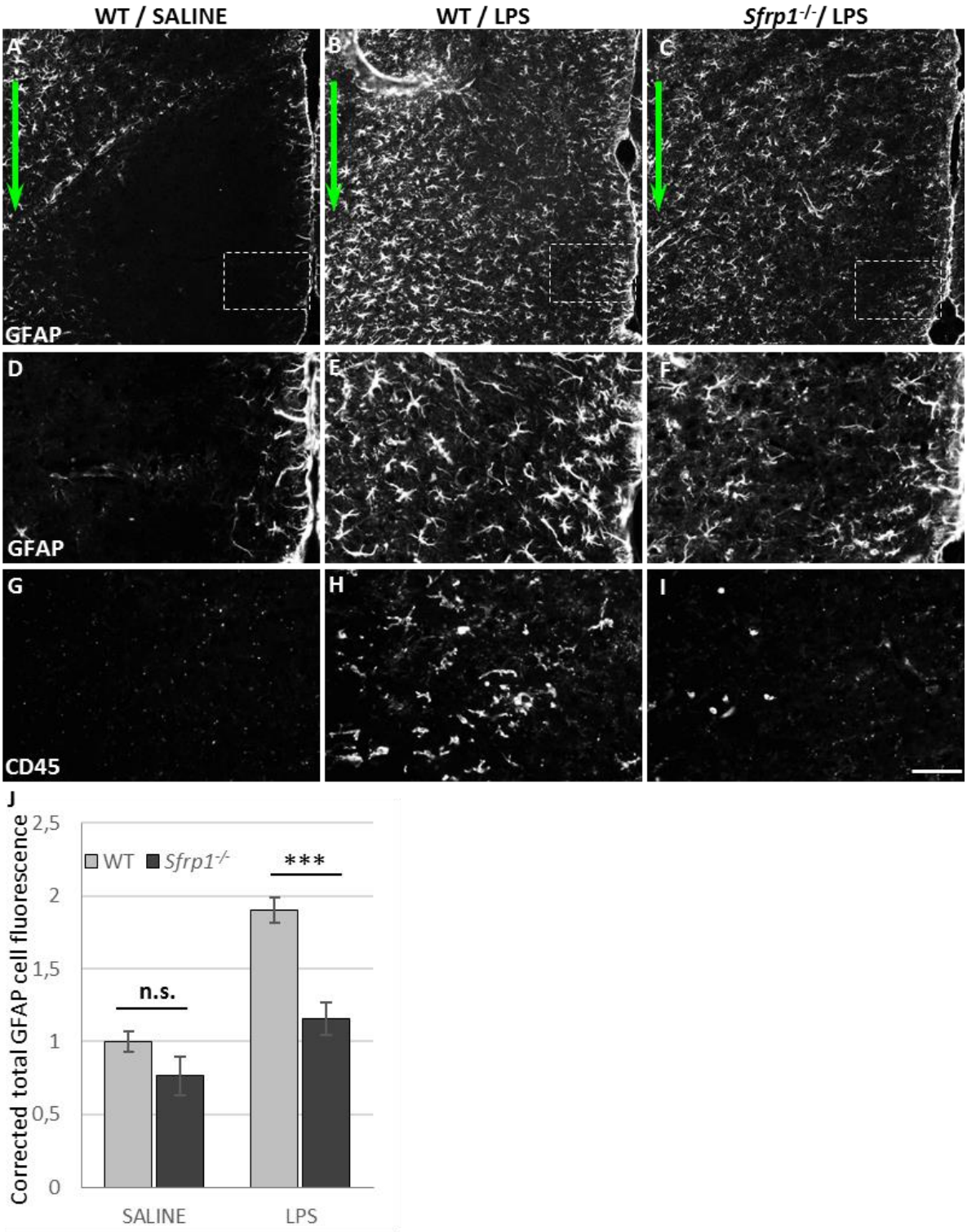


FIG.12: LPS-induced brain inflammation is reduced in *Sfrp1* null mice. Images of coronal sections from wild type (WT; A,B,D,E,G,H) and *Sfrp1*$^{-/-}$ animals (C,F,I) three days after infusion with saline (A,D,G) or LPS (B,C,E,F,H,I). Sections were immunostained with antibodies against GFAP (A-F) and CD45 (G-I). Note the increased GFAP staining in LPS treated animals (B,C,E,F) compared to saline controls (A,D). In the absence of Sfrp1 (C,F) this response is much lower. This difference in GFAP immunoreactivity was quantified using ImageJ by Corrected total cell fluorescence (J), revealing significant reduction in *Sfrp1*$^{-/-}$ mice. The decrease is more evident in the CD45 positive microglial cells in *Sfrp1* null mice (I) compared to wild type (H) LPS infused brains. Note the absence of activated microglial cells in the saline infused controls (G). Green arrows indicate the injection site. Dashed squares (in A,B,C) indicate site and size of magnification (D-I). Error bars represent Standard Error. Statistical significance: n.s. P>0.05, ***P<0.001 by Student's t-test comparing between closed. Scale bar 60µm.

In order to study further microglial activation, we analyse their morphology after intra-cortical infusion of LPS. In response to pro-inflammatory stimuli, microglial cells modify their morphology from a ramified to an amoeboid shape (Fig.13). Cell somas increases in volume and cell processes become fewer and shorter, becoming a round and compacted cell. Bipolar rod-shaped microglial cell forming strings aligned end-to-end at the damaged site have also been observed after brain injury (Au & Ma, 2017). Confocal 3D reconstruction of Iba1 immunostained wild type microglial cells showed that, compared to the saline (Fig.13A), LPS infusion triggers their processes reorganization as expected (Fig.13C). This reorganization, although not statistically significant, was less pronounced in *Sfrp1* null brains, where microglial cells (Fig.13D) were less compacted (measured by the ratio of total volume over the total area of the cell; Fig.13F) with a lower increase of the soma volume (Fig.13E). The morphology of wild type and *Sfrp1* null microglia present neither visually nor statistically significant differences in saline infused control mice.

Altogether these data support the idea that in the absence of Sfrp1 the response of glial cells to LPS is compromised, leading only to mild brain inflammation.

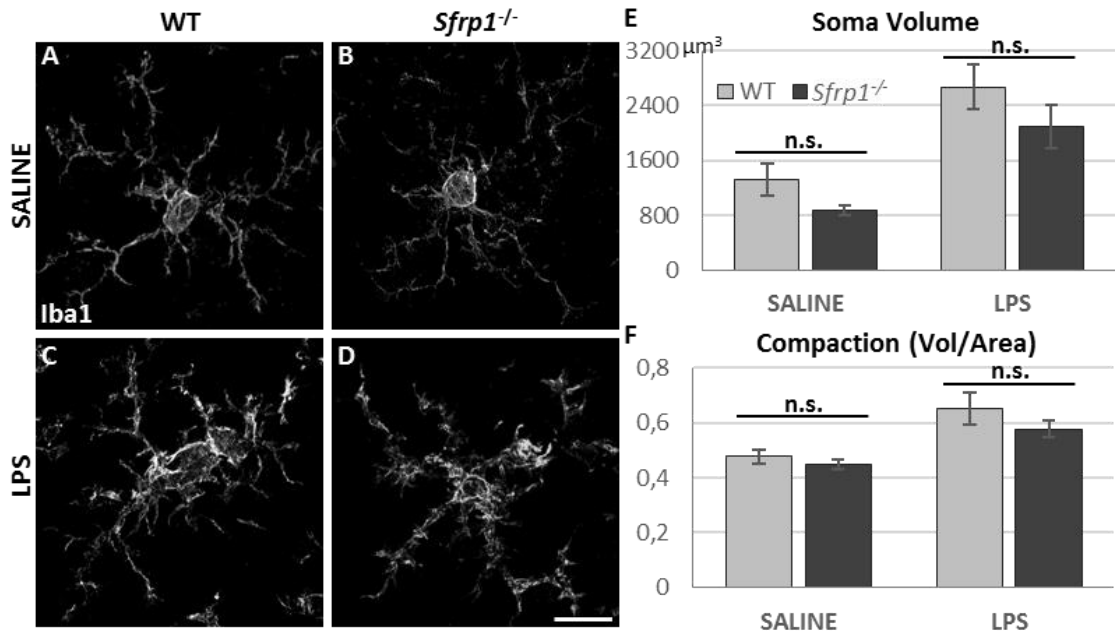


FIG.13: LPS-mediated microglial morphological changes are less evident in the absence of Sfrp1. Representative images of microglial cells from the wild type (WT; A,C) or Sfrp1$^{-/-}$ (B,D) cortex three days after saline (A,B) or LPS (C,D) intra-cortical infusion. Note that LPS induce an increase of the soma volume and a reduction of the process number and length in wild type animals (C). These alterations are less evident in the case of *Sfrp1* null microglia (B,D). Quantitation of the soma volume (E) and cell compaction (F) revealed no statistical significance between wild type and *Sfrp1*$^{-/-}$ microglia in the LPS-induced morphological changes. Error bars represent Standard Error. Statistical significance: n.s. P>0.05 by Student's t-test comparing between closed. Scale bar 20µm. n = 15 cells in 3 animals per condition.

Sfrp1 ***null mice present normal glial development***

The differential response of glial cells in *Sfrp1* null mice could be linked to an overall abnormal generation of glial cells. We first analyse the expression pattern of Sfrp1 during postnatal development (Fig. 14). Coronal sections of newborn and P20 wild type animals were immunostained with antibodies against Sfrp1 and selective markers for CNS cell types (data not shown). A strong colocalization between Sfrp1 and Iba1 was observed at P0 (arrowheads in Fig.14A), but this colocalization was absent in ramified microglial cells at P20 (arrowheads in Fig.14B). During postnatal development, amoeboid microglial cells changes their shape into ramified parenchymal microglia. This process seems to be associated with the loss of Sfrp1 expression.

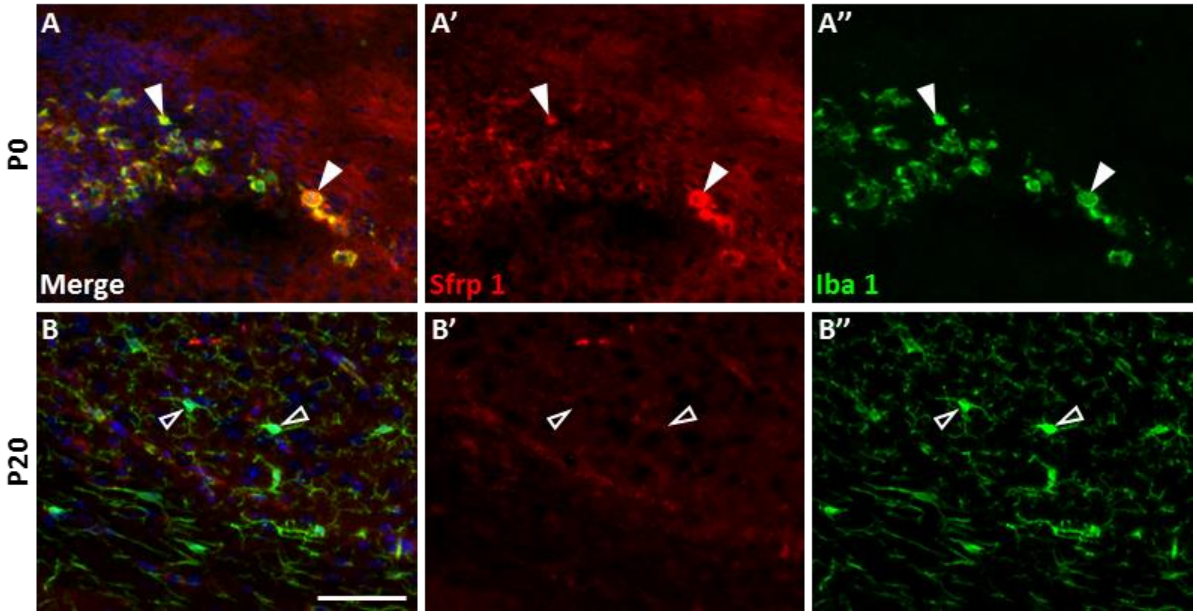


FIG.14: Sfrp1 expression decreases during postnatal development. Coronal sections of wild type P0 (A) and P20 (B) brains immunostained with antibodies against Sfrp1 and Iba1. A significant reduction of Sfrp1 expression levels is observed at P20, accompanied by the acquisition of a "resting" morphology. Arrowheads mark Sfrp1 expression in amoeboid microglial cells (A). Empty arrowheads mark ramified microglial cells (B), with no Sfrp1 expression. Scale bar 60µm.

Microglial cells originate from yolk-sac derived macrophage precursors that colonize the brain around E9 (Ginhoux et al., 2010). Following extravascular routes, such as the pia or the ventricles, they colonize the brain and expand through cell proliferation, scattering throughout the brain parenchyma (Reemst et al., 2016). Astrocytes have instead a neural origin and are generated at the end of the neurogenic wave during a variable period that depends on the brain region (Pinto & Gotz, 2007).

To discard the possibility of developmental associated glial defects in *Sfrp1* null mice, we immunostained with antibodies against Iba1 and GFAP cryostat sections from wild type and *Sfrp1* null brains at P0, P4, P8 and P20 (Fig.15). We did not observe substantial differences between the two genotypes in the organization of both types of cells.

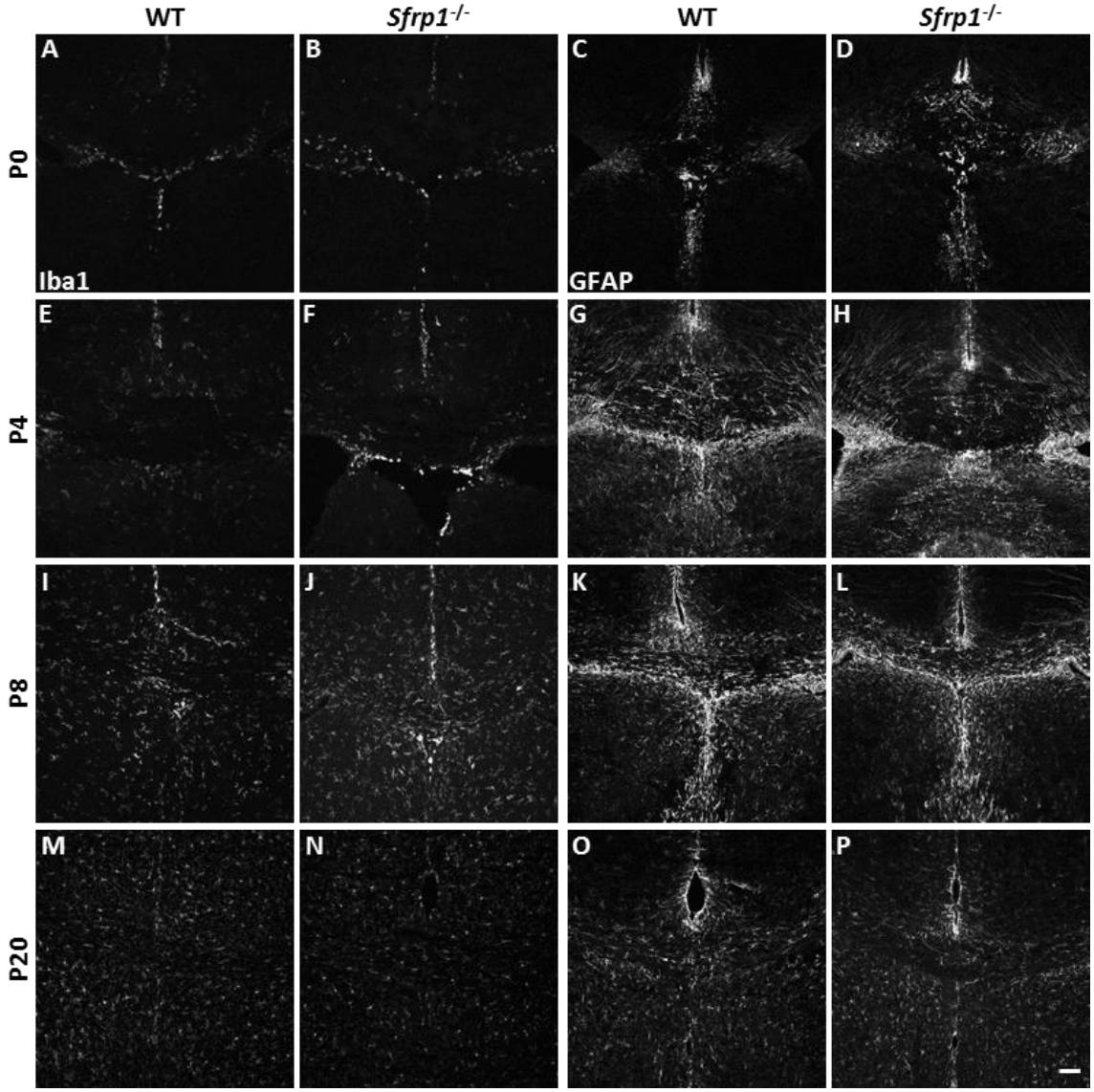


FIG.15: *Sfrp1* null mice present no apparent glial development defects. Coronal cryostat sections from wild type (A,C,E,G,I,K,M,O,Q,R) and *Sfrp1*$^{-/-}$ (B,D,F,H,J,L,N,P) animals sacrificed at birth (A-D,Q), P4 (E-H), P8 (I-L), and P20 (M-P,R) were co-immunostained for Iba1 (A,B,E,F,I,J,M,N,Q,R) or GFAP (C,D,G,H,K,L,O,P), and Sfrp1 (Q,R). No major differences were observed in number or distribution were observed between wild type and Sfrp1$^{-/-}$ microglia. Scale bar 60µm.

Sfrp1 *gene addition induces an inflammatory response*

Given that Sfrp1 effect was not apparently linked to developmental alterations, we next asked if Sfrp1 overexpression in the brain was sufficient to induce an inflammatory response, as we observed *in vitro* (Fig.16). To this end, we performed lentiviral mediated *Sfrp1* gene addition into the lateral ventricle of 10 weeks old wild type mice. We infused

lentiviral particles containing *Sfrp1*-IRES-GFP or control IRES-GFP (using GFP expression to determine the infection efficiency). As expected from the procedure, infected cells were mostly found along the wall of the lateral ventricle and in the choroid plexus (Fig.16A). In some cases, infected cells were also found along the rostral migratory stream. One month after infection, animals were sacrificed and processed to detect diverse inflammatory markers. Animals with *Sfrp1* gene addition developed a strong inflammatory response, whereas animals infected with the empty vector had no signs of inflammation (Fig.16B-I).

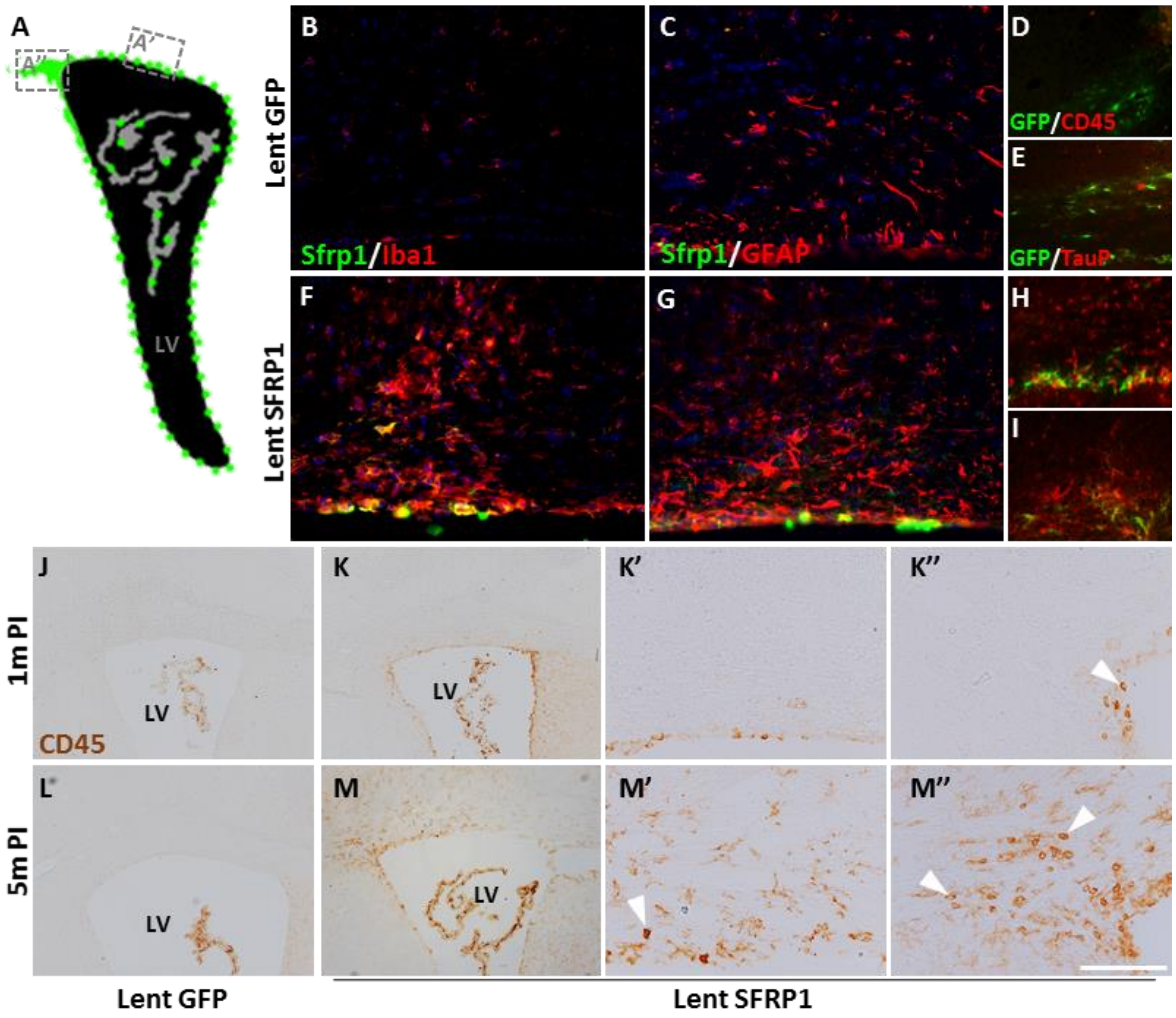


FIG.16: Sfrp1 overexpression induces an inflammatory response. A) Schematic diagram of lentiviral-mediated infection into the lateral ventricle (LV) of infused mice (infected cells marked in green), representing the position in which images in B,C,F,G,K' and M' (A'), and D,E,H,I,K'' and M'' (A'') where taken. Infected mice were sacrificed one (B-J,L) or five months (K,M) after infusion. Coronal cryostat sections of IRES-GFP (B-E,J,L) or SFRP1-IRES-GFP (F-I,K,L) infected brains were immunostained with antibodies against Iba1 (B,F), GFAP (C,G), pTau (E,I) or CD45 (D,H,J-M). Increased microglial activation (compare F,H with B,D), astrocyte reactivity (compare G with C) and tau hyper-phosphorylation (compare I with E) was observed in brains overexpressing SFRP1. Sfrp1-induced inflammatory response amplifies with time (compare M with K). Sustained overexpression of SFRP1 in the lateral ventricle leads to the infiltration of CD45 highly positive macrophages or lymphocytes into the brain parenchyma, highlighted by arrowheads (K,M). LV marks Lateral Ventricle. Scale bars 100µm.

The cortex of Lent-Sfrp1 infected animals presented a large number of reactive astrocytes, characterized by enlarged and long processes and a strong GFAP immunoreactivity (Fig.16G). Similarly, a large number of Iba1-positive activated microglial cells was detected in the surrounding of the site of infection (Fig.16F). Microglia presented an amoeboid appearance with high expression of CD45 (Fig.16H), a marker of microglial activation. Around the infected cells, there was also accumulation of hyper-phosphorylated Tau (Fig.16I), an established marker for neurodegeneration and inflammation (Grubman et al., 2016).

In order to determine whether or not this effect was transitory and the brain eventually resolved the Sfrp1 induced neuroinflammation, we analyse the effect of lentiviral infection after five months (Fig.16L,M). After such a period, inflammatory signs were still persistent and neuroinflammation had actually amplified (in Fig.16, compare M with K), in contrast to the animals infected with the empty vector (Fig.16L), which were normal. Indeed, the cortex of Lent-Sfrp1 treated animals showed a large number of CD45 positive cells, indicating the presence of activated microglial cells. Notably, in the brain parenchyma there was also a number of highly positive CD45 and round-shaped cells likely representing infiltrated macrophages or lymphocytes (arrowheads in Fig.16M' and M'').

Together these results support the idea that Sfrp1 is necessary and sufficient to trigger an inflammatory response in the brain.

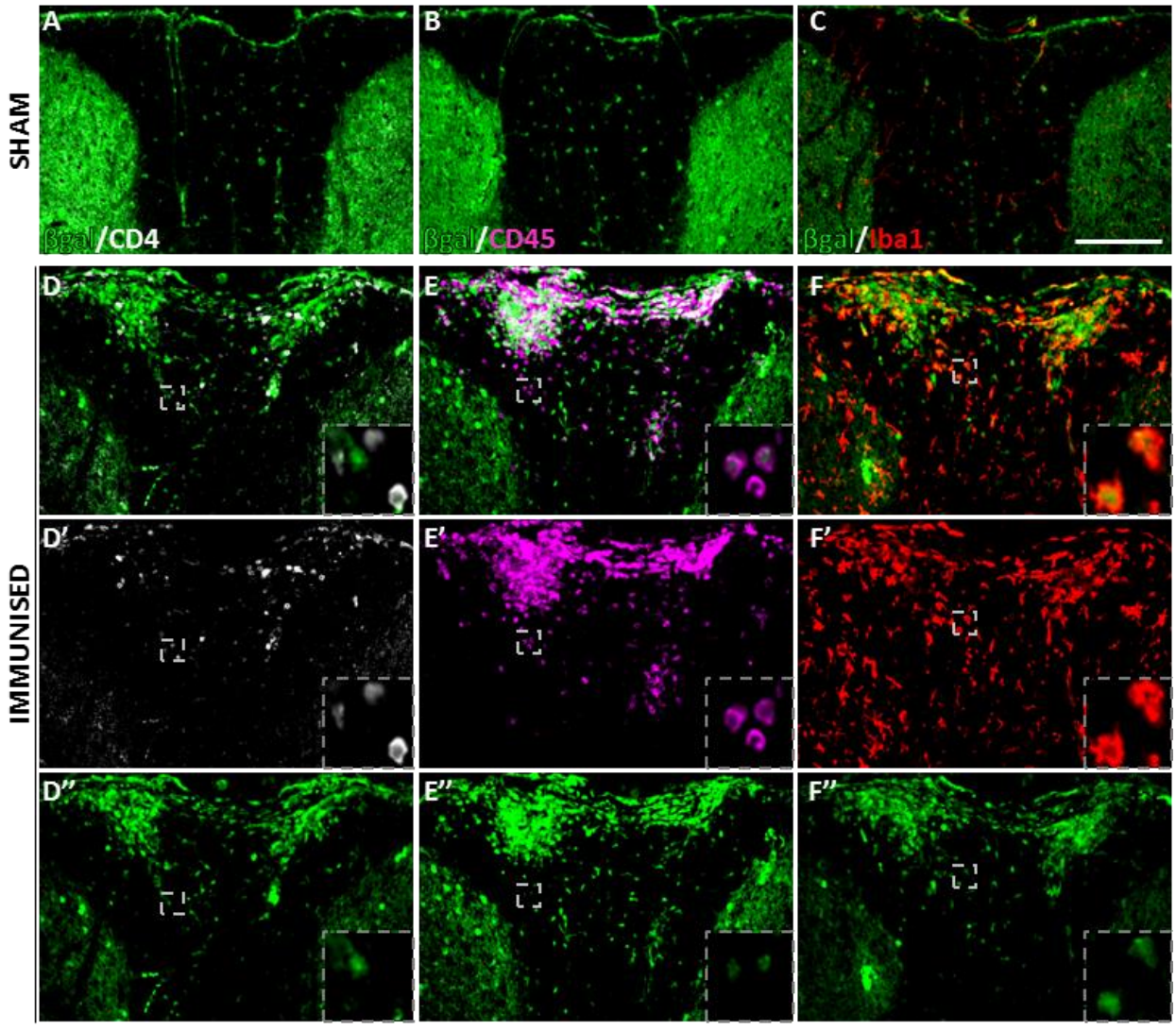
3. Sfrp1 exacerbated the symptomatology of EAE

We reasoned that if Sfrp1 has a general pro-inflammatory activity in the brain, its expression should be upregulated in other experimental paradigms with a clear inflammatory component other than that induced by LPS.

Multiple Sclerosis is a degenerative autoimmune disease with a strong inflammatory component. The principal model used to study this disease is the Experimental Autoimmune Encephalomyelitis (EAE). EAE is generated by the passive immunization of mice with Myelin Oligodendrocyte Glycoprotein (MOG) fragments, which triggers an autoimmune response. We thus used this model to test further the implications of Sfrp1 in CNS inflammation.

Sfrp1 expression is upregulated in the spinal cord of mice with EAE

We generated EAE in groups of female mice using a standardized protocol (Borroto et al., 2016) and compared them with animal treated only with Pertussis toxin, which is needed to increase BBB permeability, but has no other effects, thus serving as sham controls (Fig.17). To begin this study, animals were sacrificed 16 days after immunization, when the symptomatology of EAE reaches the highest level. To standardize the analysis, we used the spinal cord, as this is one of the most affected areas in EAE, and focused on the dorsal thoracic region occupied by the dorsal fasciculus since this is one of the regions with major infiltration of blood born cells.



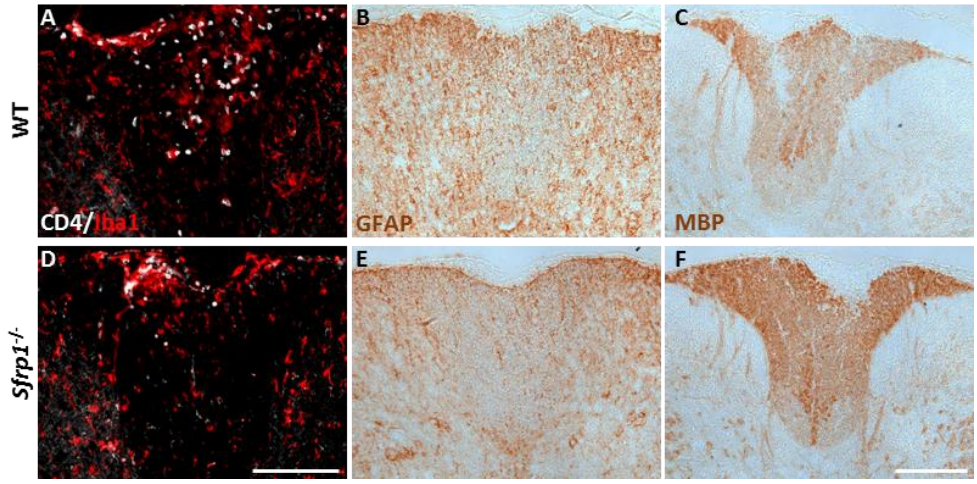
To determine Sfrp1 expression in immunised animals, we took advantage of *Sfrp1* heterozygous mice, in which a βgal cassette, useful as an expression reporter, disrupts one allele of the *Sfrp1* gene (Satoh et al., 2006). The use of βgal reporter is a great advantage as disruption of the BBB that occurs in EAE allows the infiltration of IgGs, making immunostaining with mouse immunoglobulins difficult to interpret. Furthermore, the βgal in the *Sfrp1*$^{+/-}$ animals is tagged with a nuclear location signal that simplifies the identification of the Sfrp1 source, otherwise complicated by the diffusible nature of the protein. Compared to the spinal cord of control mice (Fig.17A-C), that of EAE animals present a vast infiltration of CD4-positive lymphocytes (Fig.17D), CD45- and Iba1-positive macrophages, and an evident activation of resident CD45- and Iba1-positive microglial cells (Fig.17E,F). This inflammatory response was associated by a massive presence of βgal-positive cells (Fig.17D''-F''), not observed in controls in which βgal expression appeared to colocalise only by few Iba1-positive but CD45-negative resting microglial cells and possibly by few astrocytes (Fig.17A-C). According to βgal reporter and marker distributions, activated microglia and infiltrated macrophages (Fig.17E,F), but not CD4-positive infiltrated lymphocytes (Fig.17D), were the main source of Sfrp1.

Sfrp1 *null mice develop a milder form of EAE*

The upregulation of Sfrp1 expression in EAE prompted us to analyse if the lack of Sfrp1 could produce an attenuation of EAE pathogenesis. Wild type and *Sfrp1* null female mice were immunised according to a standard protocol (Borroto et al., 2016) and 16 days after immunization, the animals were sacrificed. Cryostat sections of the dorsal thoracic spinal cord from both genotypes were stained with antibodies against different markers to determine, besides inflammatory mediators, cell invasion and histological damage (Fig.18).

FIG.17: Sfrp1 expression is upregulated following EAE immunization. Spinal cords of *Sfrp1* heterozygous animals sacrificed after 16 days of MOG immunisation (D-F) were cryo-sectioned at the thoracic level and compared to non-immunised spinal cords (A-C). Sections were stained with antibodies against β-gal and CD4 (A,D), CD45 (B,E) or Iba1 (C,F). Images show the region of the dorsal fasciculus. Note the infiltration of CD4 positive lymphocytes (D) and CD45 and Iba1 positive macrophages (E,F), the strong activation of CD45 and Iba1 positive microglial cells (E,F). Observe the upregulation of Sfrp1, as determined by β-gal staining, in the immunised animals compared to the sham (compare A-C with D''-F''). Co-staining of nuclear β-gal reporter with different markers revealed Sfrp1 upregulation in infiltrated macrophages and activated microglia (E,F), but not in infiltrated lymphocytes (D). Scale bar 200μm.

FIG.18: Lack of Sfrp1 reduces EAE pathology. Wild type (A-C) and *Sfrp1*$^{-/-}$ (D-F) mice were immunised with MOG peptides and sacrificed 16 days post immunization. Cryostat sections of the spinal cords at the thoracic level were stained with antibodies against CD4 and Iba1 (A,D), GFAP (B,E) and MBP (C,F). Images show the dorsal fasciculus region. Compared to wild type (A,B), *Sfrp1*$^{-/-}$ mice present lower infiltration of CD4 lymphocytes and macrophages (D) and reduced astrogliosis (E). Wild type animals present demyelination of the dorsal fasciculus (C), whereas *Sfrp1*$^{-/-}$ mice show mild demyelination (F). Scale bar 200µm.



Comparison of GFAP immunostaining revealed that wild type mice had higher levels of astrogliosis than that observed in *Sfrp1* null mice (in Fig.18, compare B with E). Furthermore, *Sfrp1* null mice presented a well-defined barrier of astrocytes contacting the pial surface with few infiltrated CD4-positive lymphocytes and peripheral macrophages (Fig.18D). This was different from what observed in wild type mice that presented a disrupted pial surface (Fig.18B) and a higher number of infiltrated lymphocytes and macrophages and activated Iba1-positive microglial cells (Fig.18A). Immunostaining against MBP showed that wild type spinal cords had a pronounced myelin loss (Fig.18C), which was poorly apparent in the *Sfrp1* null animals (Fig.18F).

To determine if the milder inflammation observed in *Sfrp1* null mice was associated with an improvement of the symptomatology (Fig.19), we followed the progression of the disease in new groups of wild type and *Sfrp1* null mice immunised with MOG peptides. Clinical scores were assigned depending on the extent of the paralysis each mouse presented. Scores vary from 0 (asymptomatic animal), to 5 (moribund animal). We followed EAE progression for a month after immunization (Fig.19A). The first symptoms appeared around day 8, when the distal part of the tail becomes limp. Thereafter, there was a rapid progression of the disease until day 16, when most of the wild type animals presented hind limb paralysis. After that, there was a slow improvement of the symptoms until day 30 when, in general, half of the animals present no symptoms.

Compared to the wild type, *Sfrp1* null mice respond to immunization with a slower and less acute progression. The first symptoms of *Sfrp1* null mice also appeared at day 8, but more severe symptoms were observed much later than in wild type and rarely reached the same severity. While at day 12, wild type animals reached a 2.5 clinical score (impaired righting reflex), *Sfrp1* null animals reached this level 3 days after. At day 15, *Sfrp1* null animals reached a plateau and for 5 days no further worsening was observed. In contrast, wild type animals showed higher scores (3.5). After plateau, both genotypes slowly improved in the next 20 to 30 days with no detectable differences between genotypes. Importantly, extreme symptoms were rarely observed in *Sfrp1* null mice, and in fact, only 15% of the *Sfrp1* null animals showed them as compared to the 50% of the wild type animals (in Fig.19, compare C with B).

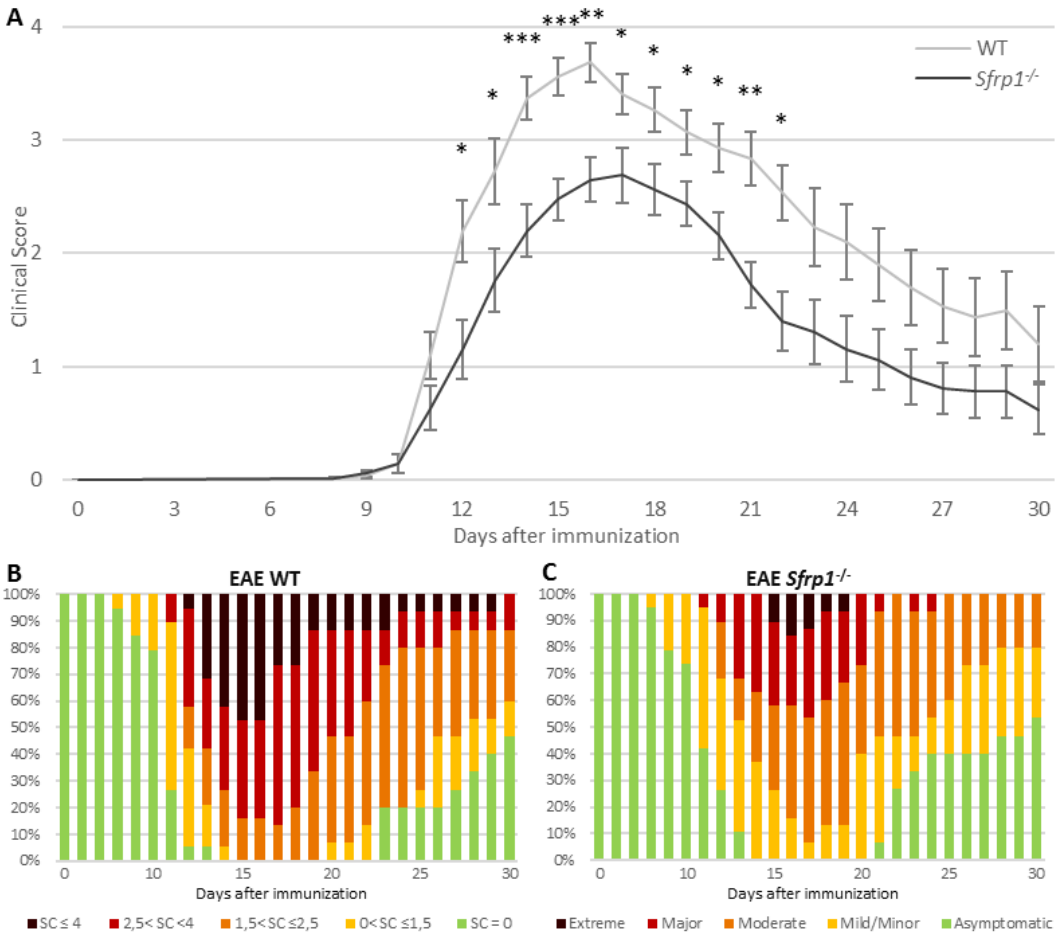


FIG.19: *Sfrp1* null mice are more resistant to clinical signs of EAE. A) Time course progression of the mean clinical score after EAE induction in wild type (light grey) and *Sfrp1* null mice (dark grey). Note that *Sfrp1*$^{-/-}$ mice have significantly milder symptoms than wild type throughout the course of the disease. B,C) Representations of the percentage of mice affected with extreme (black), major (red), moderate (orange), mild (yellow) or no symptoms (green) after EAE induction in wild type (B) and *Sfrp1* null (C) animals. Significantly, fewer animals present severe score in the *Sfrp1* null group. Error bars represent Standard Error. Statistical significance: *P<0.05, **P<0.01, ***P<0.001 by Mann–Whitney U nonparametric test comparing at same day after immunization.

Altogether, these data suggest that Sfrp1 contributes to exacerbate EAE pathology and suggest that Sfrp1 neutralization could make animals more resistant to EAE progression and severity.

4. Sfrp1 regulates microglial activation by modulating ADAM10 proteolytic activity

Altogether, the results of these studies indicate that Sfrp1 is required a prototypic inflammatory response. Sfrp1 overexpression stimulate glial cells, particularly microglial cells, likely priming their response to an insult. Given the already reported mechanisms of action, Sfrp1 could exert this this function either by interaction with the Wnt signalling pathway or by regulating ADAM10 processing activity. Alternatively, Sfrp1 could directly interact with an unknown receptor.

Sfrp1 regulates ADAM10 activity present in microglial cells

According to the literature, ADAM10 sheds a wide range of substrates that mediate inflammatory processes. We thus decided to test if Sfrp1 effects on microglial cells could be explained through this mechanism. First, we analysed whether in microglial cells cultures ADAM10 activity is modulated by Sfrp1, given the ubiquitous expression of ADAM10 protein (Y. Zhang et al., 2014). To this end, we took advantage of a fluorogenic peptide that contains a sequence recognised and cleaved by ADAM8, ADAM9, ADAM10 and ADAM17. Primary microglial cells from wild type and *Sfrp1* null mice were incubated with this peptide for four hours and the tissue culture medium was collected to determine the generated fluorescent signal, indicative of the degree of enzymatic activity present in the cells (Fig.20.A). *Sfrp1* null microglia showed a 50% increase in ADAM enzymatic activity with respect to that of wild type cells, and addition of a selective ADAM10 inhibitor or of human recombinant SFRP1 protein decreased of about a 30% ADAM activity (Fig.20B). This indicates that Sfrp1 modulates ADAM functions in microglial cells.

Once verified that microglial ADAM10 activity is regulated by Sfrp1, we sought to analyse if neuroinflammatory regulators processed by ADAM10 are differentially proteolysed in the presence of Sfrp1. Among them, ADAM10 sheds at least three transmembrane proteins that have been implicated in silencing and reshaping the adverse innate immune response: TREM2 and the ligands of the ligand/receptor pairs

CD200/CD200R and CX3CL1/CX3CR1. Unfortunately, the processing of these molecules *in vivo* is difficult to determine, owing to their low expression and/or the lack of good antibodies. We thus decided to perform our analysis *in vitro*. The CD200 and CX3CL1 ligands are constitutively expressed in neurons and are well-recognised substrates of ADAM10 (Hundhausen et al., 2007; Wong et al., 2016). We generated double-tagged versions of their coding sequence and transfected the corresponding plasmids in the HEK293 cell line. After determining transfection efficiency, cells were treated with a selective inhibitor of ADAM10 or SFRP1 recombinant protein. After 24 hours of treatment, the tissue culture media was collected and the presence of shed N-terminal fragments analysed by Western Blot. Notably, the presence of the ADAM10 inhibitor or of SFRP1 decreased the processing of both CD200 (Fig.20D) and CX3CL1 (Fig.20C), suggesting that Sfrp1 modulation of neuroinflammation could involve the regulation of both CD200/CD200R and CX3CL1/CX3CR1 signalling.

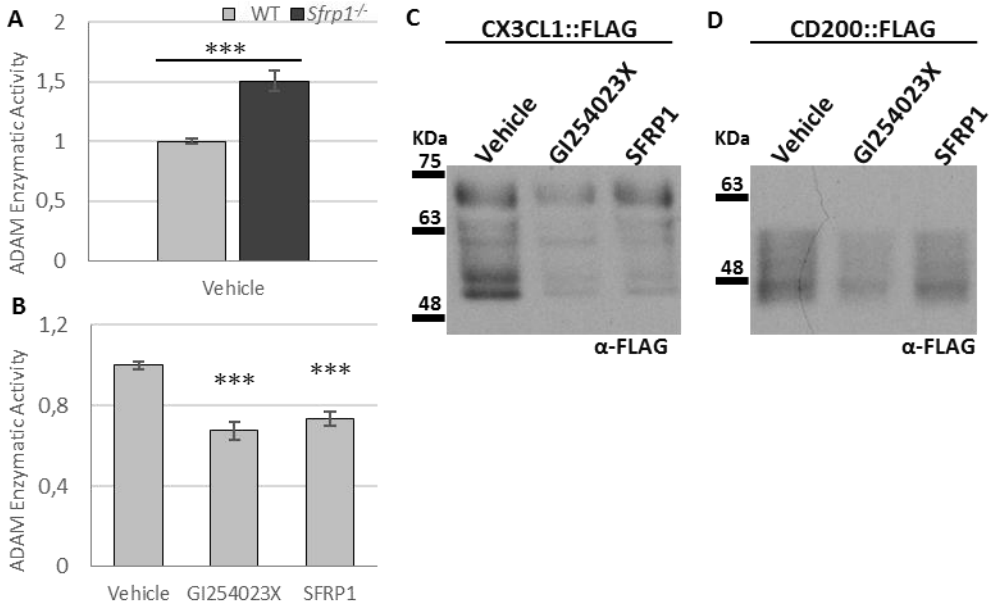


FIG.20: Sfrp1 modulate ADAM10 shedding of CX3CL1 and CD200 in culture. A,B) Fluorometric determination of ADAM enzymatic activity in purified microglial cultures. ADAM activity was normalized to untreated wild type microglia levels. ADAM proteolytic activity was measured in wild type (light grey) and *Sfrp1* null (dark grey) primary microglia (A). Note the increased activity in the absence of Sfrp1. The effect of 0,5µg/ml Sfrp1 or 5µM ADAM10 specific inhibitor (GI254023X) was analysed on wild type purified microglial cultures (B) with similar results in decreasing ADAM enzymatic activity. The effect of these treatments was tested in HEK293 transfected with N-terminal FLAG tagged CX3CL1 (C) or CD200 (D). After 24 hours treatment with 0,5µg/ml Sfrp1 or 5µM ADAM10 inhibitor (GI254023X), the presence of CX3CL1 (C) or CD200 (D) soluble forms were analysed by Western Blot to detect FLAG epitope on cell supernatants. Both proteins are highly glycosylated and therefore run as a smear in SDS-PAGE gels. Error bars represent Standard Error. Statistical significance: ***P<0.001 by Student's t-test comparing with vehicle treated wild type.

Sfrp1 potentiates ADAM10 shedding of TREM2

TREM2 is a receptor present in the membrane of microglial cells and involved in their phagocytic activity and response to neuroinflammation (Colonna & Wang, 2016). To determine if its processing could be also regulated by Sfrp1, we took advantage of a stably transfected HEK293 FLP-in line that express a tagged form of hTREM2 (Kleinberger et al., 2014). Cells were treated with two different inhibitors of ADAM10 or with SFRP1 recombinant protein for 24 hours, when media were harvested and analysed (Fig.21). As previously reported (Kleinberger et al., 2014), treatment of hTREM2 expressing cells with either a selective inhibitor of ADAM10 or a broader ADAM inhibitor highly reduces the generation of soluble TREM2 of about 70%. Unexpectedly, SFRP1 treatment enhanced TREM2 shedding about five folds compared to vehicle treated cells (Fig.21B). This indicates that Sfrp1 favour the generation of sTREM2, which in turn has been shown to polarize microglial cells towards a pro-inflammatory state (Zhong et al., 2017).

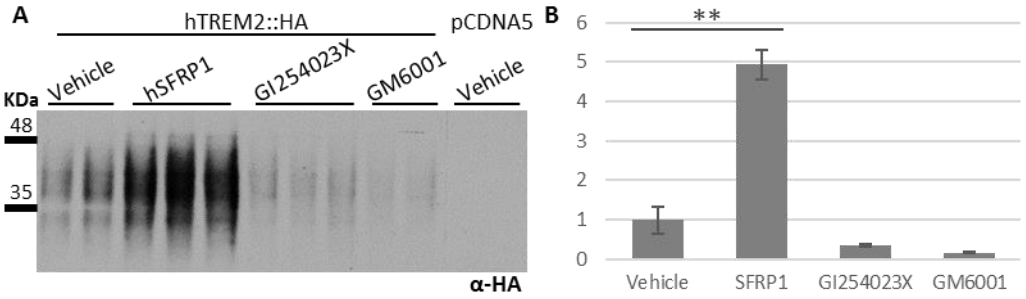
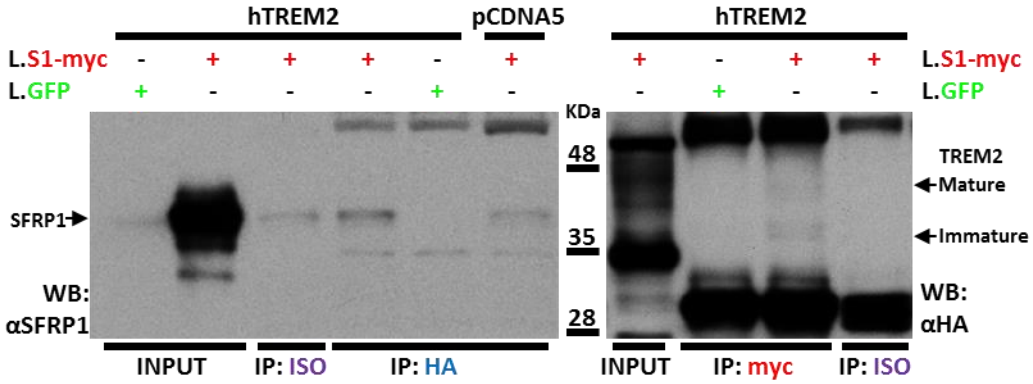


FIG.21: SFRP1 promotes TREM2 shedding. hTREM2 shedding was analysed in HEK293 FLP-in cells stably transfected with hTREM2 with a N-terminal HA tag. Cells were treated for 24 hours with 0,5µg/ml Sfrp1, 5µM ADAM10 selective inhibitor (GI254023X), or 25µM ADAM broad inhibitor (GM6001). After treatment, the content of soluble TREM2 levels in cell supernatants were analysed by Western Blot to detect HA epitope (A) and subsequently quantified (B). SFRP1 induces a five folds increase in soluble TREM2 levels. HEK293 FLP-in pCDNA5 were used as negative control. Error bars represent Standard Error. Statistical significance: **P<0.01 by Student's t-test comparing with vehicle treated.

In the attempt to elucidate how Sfrp1 could enhance TREM2 shedding, we hypothesised that Sfrp1 could directly interact with TREM2, thereby facilitating ADAM10 processing. To test this possibility we infected the stable hTREM2 expressing cell line with lentiviral particles containing Sfrp1 and analyse if the two proteins co-immunoprecipitated (Fig.22). Preliminary results indicate that this interaction is taking place, allowing us to propose a working model in which interaction of Sfrp1 with both ADAM10 and TREM2 could near enzyme and substrate facilitating the shedding.

FIG.22: SFRP1 interacts with hTREM2. Stable HEK293 FLP-in cell line expressing hTREM2 with an N-terminal HA tag was infected with lentiviral particles carrying SFRP1-myc or GFP constructions. HEK293 FLP-in pCDNA5 were used as negative control. Co-immunoprecipitation of different combinations described were performed with antibodies against hTREM2 HA tag (left panel) or SFRP1 myc tag (right panel), and with isotype controls (ISO). Interaction was analysed by Western Blot to detect SFRP1 (left panel) or HA (right panel). SFRP1 and hTREM2 seems to interact, although part of this co-precipitation might be unspecific.



5. Sfrp1 neutralization as therapeutic target

Sfrp1 immunosuppression counteracts AD progression

Altogether our studies raise the interesting possibility that SFRP1 could represent a valuable target to counteract the undesirable effects of neuroinflammation in different neurodegenerative disease. To obtain the proof of concept that this could be an avenue worth pursuing, we decided to use a SFRP1 blocking antibody (Fig.23A), generated in collaboration with Mercedes Dominguez (ISCIII, Majadahonda), to neutralize Sfrp1 activity in a mouse model of AD.

Two months old APP;PS1 animals were injected through retro-orbital sinus with 100ug of anti-SFRP1 or a control IgG isotype for two months, once a week (Yardeni et al., 2011). After treatment, animals were perfused and histologically analysed. Animals treated with anti-SFRP1 but not with IgGs, present accumulations of IgGs in the cortex (Fig.23D,E), in part associated with the Aβ-positive amyloid plaques where Sfrp1 also localizes (Esteve et al., in preparation). Thioflavin S staining (Fig.23B,C) revealed reduction (about 50%; Fig.23H) of Aβ plaque deposits in anti-SFRP1 treated animals, although changes did not reach statistical significance. This suggests that neutralising Sfrp1 function likely diminish Aβ plaque burden. In addition, treatment with anti-SFRP1 antibodies significantly increased the recruitment of Iba1 positive microglial cells around the Aβ positive plaques (Fig.23F,G). Microglial cells have an enhanced phagocytic activity

when recognise Fc domain of IgGs (Xiang et al., 2016). We thus speculate that anti-SFRP1 antibodies localised to the plaques, could favour the phagocytic activity of microglial cells, thereby increasing the clearance of Aβ plaques, an effect not observed with control IgG. Together these results suggest that, at least in AD model, the treatment with anti-SFRP1 might have a beneficial effect with a dual mechanism: prevention of plaque formation and enhancement of plaque clearance.

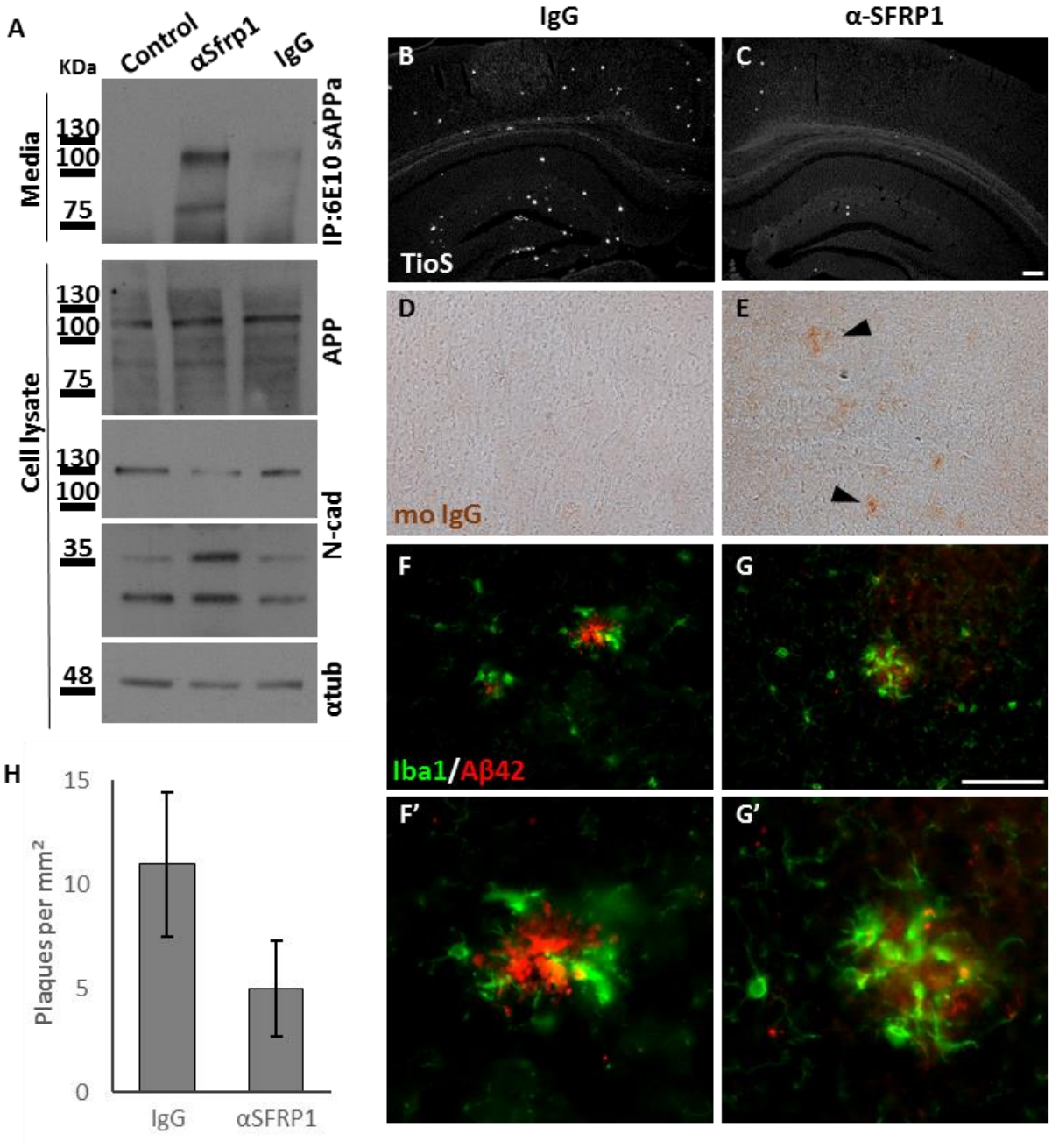


FIG. 23: Antibody mediated neutralization of Sfrp1 activity ameliorates Aβ plaque burden. Western Blot analysis of telencephalon dissociated cultures (A) indicates that impaired Sfrp1 activity favours non-amyloidogenic processing of APP. Telencephalon from E13.5 wild type embryo was dissociated and cultured for 3 days in the presence of an antibody against SFRP1 (lane 2) or control isotype IgGs (lane 3). Media were immunoprecipitated with an antibody against Aβ and analysed by Western Blot (top panel). Note increased levels of sAPPα in media from αSFRP1 treated cultures. Culture lysates were analysed for APP (second panel) and N-Cadherin processing (third panel). Note that total N-cadherin levels (135KDa) were decreased, whereas the C-terminal fragment (35KDa) was increased incultures treated with αSFRP1. Coronal sections of the frontal cortex of four month old APP;PS1 male mice systematically perfused for two months with an antibody against Sfrp1 (C,E,G) or an IgG isotype control (B,D,F) were stained with Thioflavin S (TioS; B,C) or immunostained with antibodies against mouse IgGs (D,E) or Iba1 and Aβ42 (F,G). Note the decrease in Aβ plaque burden in the animals treated with Sfrp1 antibody (B,C) with strong tendency in quantification (H) VS compared to those treated with IgG. Although due to huge inter-variance and reduced animals used (IgG n=4; αSFRP1 n=8) there are no statistical significance (H). In the cortex of animals treated with anti-Sfrp1, accumulations of mouse IgGs are observed (marked with arrowheads in E) in the frontal cortex, where are no detected in IgG treated mice (D), suggesting specific accumulation of anti-Sfrp1 in Aβ plaques, binding to Sfrp1 where is accumulated. Treatment with the antibody against Sfrp1 induces higher clustering of microglial cells surrounding Aβ plaques (F,G). Scale bars 100μm.

DISCUSSION

Neurodegenerative disorders have, in general, a multifactorial aetiology but for all of them neuroinflammation is one of the central processes that amplifies the primary damage. The primary origin even of Multiple Sclerosis (MS), considered the prototypic immune-mediated CNS disease, is now controversial. In fact, it has been proposed that the primary cause of the disease is a neurodegeneration in which uncontrolled neuroinflammation triggers neuronal damage, whereas inflammatory demyelination represent a secondary consequence (Trapp & Nave, 2008). Growing evidence suggests that dysfunctional or aberrantly activated glial cells can be a primary cause rather than a consequent reactive contributor of neurodegenerative disorders (Ransohoff, 2016). Thus, understanding neuroinflammatory processes might provide important clues to modulate the glial-driven cascade of neurodegenerative disorders with beneficial results.

In this thesis, we have provided evidence for a direct implication of Sfrp1 in neuroinflammation. Indeed, Sfrp1 is strongly upregulated in activated microglial cells and infiltrated macrophages and, to a lower extent, in reactive astrocytes, in all the experimental conditions in which neuroinflammation was induced (LPS, AD or EAE). Conversely, in the absence of *Sfrp1*, the response of glial cells to pro-inflammatory stimuli was very reduced, with an associated decrease of the pathological traits and symptoms of the diseases. Furthermore, the addition of *Sfrp1* to the cortex was sufficient to promote the activation of glial cells, inducing an inflammatory response that triggered the infiltration of immune cells. More importantly, in initial studies we have shown that neutralization of Sfrp1 function with blocking antibodies alleviated the pathological traits of AD. This indicates that neutralizing Sfrp1 pro-inflammatory activity may open a new therapeutic avenue to treat neurodegenerative diseases and related disorders with an associated neuroinflammatory component.

From a mechanistic point of view, our data indicate that Sfrp1 may regulate neuroinflammation through ADAM10, in line with our previous studies showing that Sfrp1 modulates ADAM10 proteolytic activity. Indeed, microglial cells present a Sfrp1-modulated ADAM10 activity, and, at least *in vitro*, this activity seems to regulate the

proteolysis of relevant immune modulators, such as microglial TREM2 and neuronal CD200 and CX3CL1, in a substrate-dependent manner.

1. Sfrp1 promotes a reactive state of microglial cells

The observation that motivated our study was the strong decrease of gliosis surrounding the deposits of Aβ in the brain of a mouse model of AD, when *Sfrp1* expression was genetically inactivated. Aβ plaque burden is reduced in the absence of *Sfrp1* and this could explain the mild gliosis observed in the APP;PS1;*Sfrp1*$^{-/-}$ mice model. However, gliosis was mild also surrounding the few Aβ plaques present in the APP;PS1;*Sfrp1*$^{-/-}$ pointing to the possibility that Sfrp1 could directly impinge upon inflammatory events.

Sfrp1 contributes to exacerbate neuroinflammatory responses

It is important to remark that the absence of Sfrp1 does not completely impair the inflammatory response. This is an important observation, because, as previously mentioned, an acute inflammatory response is a necessary reaction to damage and has beneficial functions because it promotes pathogen elimination, tissue repair and restore CNS homeostasis (Sochocka et al., 2016). Both LPS-infused and MOG-immunised *Sfrp1* null animals develop an inflammatory response, but this response was milder than that of control animals and likely sufficient to neutralize the proinflammatory trigger and thus prevent a chronic inflammatory state. This effect was particularly remarkable in limiting the severity of the clinical symptoms of EAE, where we observed that only 15% of the *Sfrp1* null animals presented severe symptoms, in contrast to the 50% of the wild types. Although, EAE progression is triggered by the infiltration of peripheral macrophages and lymphocytes (Ajami et al., 2011), microglial activation is required for the development and maintenance of inflammatory lesions associated to EAE (Ding et al., 2014), likely favouring further infiltration of peripheral immune cells (Heppner et al., 2005). Although, we believe that the EAE phenotype observed in Sfrp1-/- mice is likely linked to a decreased activation of local microglila cells, we cannot discard that this might be also associated to less reactive infiltrated macrophages, which also seem to express *Sfrp1* during EAE.

We have shown that there is a difference in the cytokine secretion profile in mixed glial cultures from *Sfrp1* null and the wild type mice after an LPS pro-inflammatory stimulus. We believe that the difference is mostly due to a lower primary response of microglial cells, because it has been shown that microglial cells mediate the response of astrocytes to LPS (Holm et al., 2012). Taking this into account, the attenuated phenotypes of *Sfrp1* null mice could reflect an attenuated activation of microglial cells. A principal role of microglial cells, rather than of astrocytes is also supported by the fact that Sfrp1 has been implicated in other diseases with a chronic inflammatory component that do not affect the CNS, and therefore where astrocytes are not present.

Another reason to think that Sfrp1 activity has a preponderant role on microglia rather than astrocytes is the poor morphological changes of *Sfrp1* null microglia in response to LPS. These mild changes, observed both *in vitro* and *in vivo,* are likely more relevant than what our analysis, limited to a low number of cells, now seems to show. Although not statistically significant, our study shows that in absence of *Sfrp1,* microglial cells tend to be less compacted in response to LPS, which has important implications in the course of neuroinflammation. For example, we have observed that the absence of *Sfrp1* the secretion of anti-inflammatory cytokines, such as IL4, is significantly increased, although there is little effect on pro-inflammatory cytokines . This might favour *Sfrp1* null microglia polarization towards an anti-inflammatory phenotype, predisposing to tissue recovery.

Less clear is the possible significance of the observed increase of IL10, another anti-inflammatory cytokine studied. Indeed, IL10 function is rather controversial. On one side IL10 has been shown to promote healing and repair and to resolve the inflammatory phase (Shichita et al., 2014). On the other side, IL10 seems to have a detrimental role in AD, because its overexpression accelerated the pathology whereas IL10 inactivation has the opposite effect (Chakrabarty et al., 2015; Guillot-Sestier et al., 2015). The results of our study, showing that in both basal and stimulated *Sfrp1* null cells IL10 levels are increased, agrees with the view that IL10 has a beneficial role in inflammation. A possible explanation to this controversy might be linked to the levels of expression. In the AD study, IL10 concentration was rather elevated when compared to our values. Therefore,

lack of Sfrp1 with an associated moderate increase of IL10 could be beneficial to restrain the effect of a pro-inflammatory stimulus (Lobo-Silva et al., 2016).

Sfrp1 may prime microglia cells predisposing them to activation

In good agreement with the anti-inflammatory phenotype of *Sfrp1* null microglia, Sfrp1 addition or overexpression is sufficient to induce a strong inflammatory response. The treatment of glial cultures with recombinant Sfrp1 protein induces the activation of microglial cells and the secretion of cytokines. This is also observed after lentiviral-mediated *Sfrp1* gene addition in the cortex of wild type mice. The overexpression of Sfrp1 induced a strong neuroinflammatory response that persists and amplifies with time, acting as a recruitment signal for immune cells that infiltrates into the brain parenchyma. Therefore loss and gain of Sfrp1 function suggest that elevated levels of Sfrp1 may induce a hyper-reactive state of microglial cells, diminishing their activation threshold.

This process is known as “microglial priming”. This phenomenon consists in an exaggerated or increased sensitivity of microglial cells to a secondary inflammatory stimulus. Microglial priming is associated with altered morphology, upregulation of cell surface antigens and increase microglial proliferation (Perry & Holmes, 2014). Mutations or alterations in receptors or ligands of pathways related to microglial surveillance induce a constitutively primed state of microglial cells, as discussed in another section.

Recently, in a genome-wide analysis of microglia from diverse brain regions, Sfrp1 was identified as one of the genes enriched in cerebellar and hippocampal microglia (Grabert et al., 2016). This work described a marked difference between cortical and cerebellar microglia, so that cerebellar and hippocampal microglia have an enhanced immune-alert state, accompanied by a higher expression of genes involved in energy metabolism. This is in line with previous studies showing that white matter microglia have a less quiescent basal state than grey matter microglia, since the cerebellum is enriched in white matter (Hart et al., 2012).

The observation that Sfrp1 is enriched in a microglial cluster characterised by an activated immune-phenotype supports the possibility that Sfrp1 may be directly responsible for an activated or primed state of microglial cells. The same study reported

that microglia aging is region-dependent, with cerebellar microglia being the more prone to aging, because they are in immune-alert state with a higher expression of immune amplifying genes (Grabert et al., 2016). This points to a possible implication of Sfrp1 in making microglia more vulnerable to aging and age-related diseases owing to alertness well above that of other regions.

2. Sfrp1 modulates microglial activity through ADAM10

Our data together with the literature mentioned above support the idea that Sfrp1 tunes microglial activation. High levels of Sfrp1 could lead to major dysregulation of microglial functions and subsequently induce severe alterations of CNS homeostasis that result in the development of CNS pathologies. Given the multifunctional activities of Sfrp1 (Cruciat & Niehrs, 2013), there are several mechanisms by which Sfrp1 may modulate microglial activity: the first could involve the Wnt signalling pathway; the second the regulation of ADAM10 proteolytic activity or, as a third possibility, it might involve the interaction with a previously undescribed receptor.

Sfrp1 can modulate the Wnt signalling pathway in both a positive and negative way (Esteve & Bovolenta, 2010). Disruption of the Wnt signalling pathway has been implicated in some of the alterations that characterize AD (Godoy et al., 2014) and higher Sfrp1 levels could sequester Wnt ligands, reducing pathway activity (Kawano & Kypta, 2003), thereby modulating microglial response (Halleskog & Schulte, 2013). We cannot discard this possibility but we have evidence that in APP;PS1 mice that lack Sfrp1 Wnt/β-catenin signalling is not particularly affected, since qPCR analysis of the total brain mRNA levels of Axin2, a read-out of Wnt pathway activity (Nusse & Clevers, 2017), revealed no significant differences with those of the APP;PS1 mice alone.

Sfrp1 modulates processing of ADAM10 immune-related substrates

The other mechanism by which Sfrp1 could modify microglial activity implicates the negative modulation of ADAM10 (Esteve et al., 2011a). ADAM10 is implicated in neuroinflammatory and neurodegenerative events (Saftig & Bovolenta, 2015). Our study shows that Sfrp1 can modulate ADAM10 activity in microglia (Fig.20). Among the wide range of ADAM10 substrates, there are mediators of the microglial response, such

as CX3CL1 (Hundhausen et al., 2007), CD200 (Wong et al., 2016) and TREM2 (Kleinberger et al., 2014). All of them are required to maintain CNS homeostasis and microglia cells in a quiescent state (Colonna & Butovsky, 2017). Mutations in the genes encoding these regulators have been associated with susceptibility to develop neurodegenerative diseases (Kierdorf & Prinz, 2013). It is therefore plausible that high levels of Sfrp1 could disrupt their signalling, leading to an exacerbated response of microglial cells, as observed upon Sfrp1 overexpression.

CX3CL1, CD200 and TREM2 have different characteristics. CX3CL1 and CD200 are ligands, present in membrane-anchored and soluble forms, which are expressed mostly by neurons (Hughes et al., 2002; Koning et al., 2009). Their different forms interact with their respective receptors (CX3CR1 and CD200R) expressed in microglial cells (Jung et al., 2000; G. J. Wright et al., 2000), maintaining microglial cells in a quiescent state. If ligand-receptor interaction, and thus its signalling, is lost, neuronal damage appears together with microglial neurotoxicity (Cardona et al., 2006; Lyons et al., 2007). We have demonstrated that Sfrp1 impairs ADAM10-mediated shedding of both CX3CL1 and CD200 *in vitro*. Therefore, Sfrp1-modulated shedding of these neuronal ligands could limit their binding to the receptors on microglial cells, thereby allowing microglial activation (since microglia are no longer kept in a quiescent state). This mechanism could explain why Sfrp1 induced a primed or less quiescent microglia state.

The other ADAM10 substrate we have studied is TREM2. Differently from the other two, this receptor is expressed by microglial cells (Colonna & Wang, 2016). Polyanions, phospholipids and lipoproteins can bind and activate TREM2. Upon ligand binding, TREM2 activates an intracellular signal that promotes phagocytosis (Hsieh et al., 2009), survival (Otero et al., 2009) and attenuates microglial activation (Turnbull et al., 2006). At the cell membrane, TREM2 activity is regulated by ADAM10 and ADAM17-mediated proteolytic processing (Kleinberger et al., 2014). Aberrant TREM2 signalling has been related to malfunction and senescence (Zheng et al., 2017) of microglial cells and neurological disorders (Ulrich et al., 2017).

In contrast to what possibly expected, here we have shown that Sfrp1 enhances TREM2 shedding, promoting the generation of soluble TREM2 (sTREM2). The functions and interactions of TREM2 are still highly controversial, and even more debated is the

role of sTREM2. In a mouse model of AD, haploinsufficiency of TREM2, which presumably lead to lower levels of active TREM2 at the plasma membrane, have been shown to promote microglial dysfunction and to induce severe axonal dystrophy (Y. Wang et al., 2015; Y. Wang et al., 2016). sTREM2 enhances microglial survival and is sufficient to trigger pro-inflammatory responses (Zhong et al., 2017). Thus, Sfrp1-promoted processing of TREM2 could have two detrimental effects. Both effects correlate with those observed upon Sfrp1 overexpression, meaning an increase of inflammation.

Notably, reduced function of TREM2 impairs microglial survival disrupting β-catenin stabilization (Zheng et al., 2017). As stabilization of β-catenin allows its translocation to the nucleus and thus its function as final effector of the Wnt/β-catenin pathway, the shedding-mediated modulation of TREM2 activity may be an indirect mechanism with which Sfrp1 could interfere with β-catenin induced gene transcription in microglial cells. If this were the case, a possible explanation for the absence of changes in *Axin2* mRNA levels observed in AD mice might be the small proportion that microglial *Axin2* might represent over the total brain content. This establishes another level of Sfrp1-dependent regulation to Wnt ligands activity that could explain their dual and context-dependent effect on microglial response (Halleskog et al., 2011; Halleskog & Schulte, 2013; B. Li et al., 2011).

Sfrp1 modulates ADAM10 activity in a substrate-specific manner

The different effect of Sfrp1 on ADAM10-mediated shedding of different substrates indicates that Sfrp1 function might depend on the nature of the substrate. We have proposed that the Sfrp1 CRD could interact with the CRD present in ADAM10. This CRD dimerization could bring ADAM10 catalytic domain close to the Sfrp1 NTR domain, which is known to share similarities with TIMP inhibitors (Mott et al., 2000). Thereby, Sfrp1 NTR would interfere with ADAM10 enzymatic activity by allosteric competition with diverse substrates.

According to a database of predicted human protein-protein interactions (McDowall et al., 2009), TREM2 has high probability of interacting with Netrin1. Netrin1 shares

conformational similarities with the NTR domain of Sfrp1 (Banyai & Patthy, 1999). Therefore, it is tempting to speculate that the NTR domain of Sfrp1 could interact with TREM2, providing a hypothetical explanation of why Sfrp1 seems to act as positive modulator of ADAM10-dependent TREM2 processing. In this case in fact, the NTR domain of Sfrp1 will be no longer able to interfere with ADAM10 catalytic activity. Rather, by binding to ADAM10 through its CRD domain and to TREM2 by the NTR, it will bring together ADAM10 and TREM2, thus facilitating TREM2 shedding (Fig. 24).

This is not the only possibility to explain Sfrp1-enhanced shedding of TREM2. TREM2 can be shed also by ADAM17 (Kleinberger et al., 2014), the activity of which is promoted under pro-inflammatory stimuli (Thorp et al., 2011). In this case, by inducing neuroinflammation, Sfrp1 would increase ADAM17 activity and thus TREM2 shedding. This possibility needs however to be explored.

Altogether these data shows that Sfrp1/ADAM10 functional interaction is key to induce microglial activation by the modulation of ADAM10 proteolytic profile, with a mechanism that might promote or interfere the shedding of the substrate in a substrate-specific manner, although this last possibility needs to be further explored.

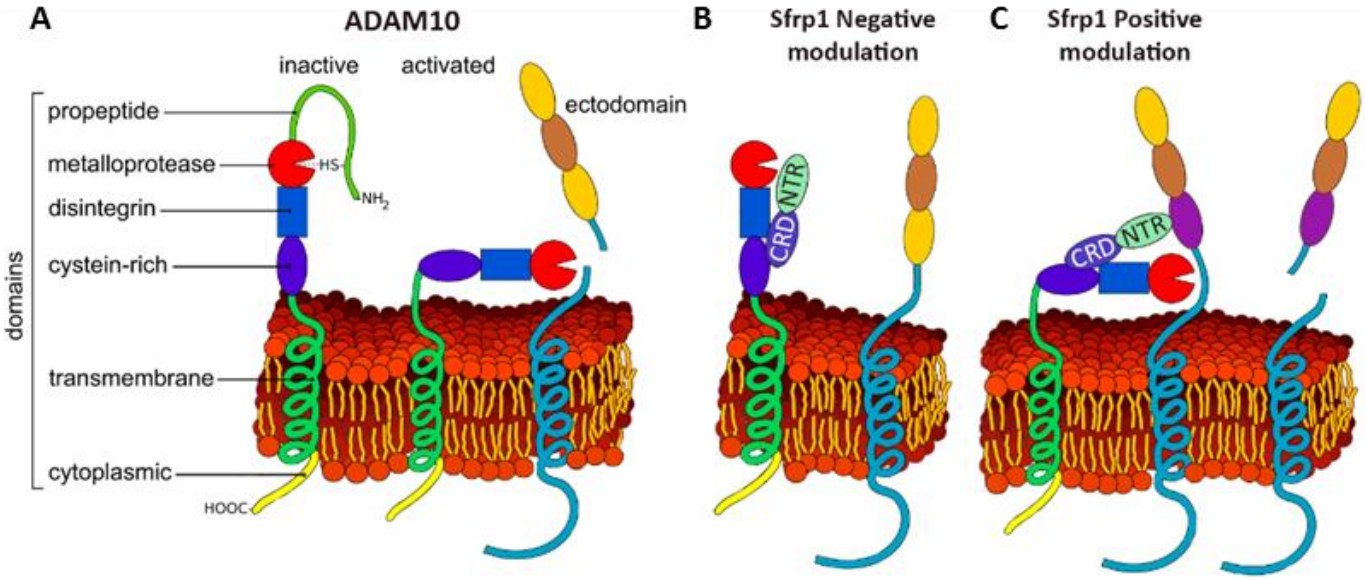


FIG.24: Proposed model for SFRP1 substrate-specific modulation of ADAM10. A) ADAM10 transmembrane metalloprotease sheds a wide range of substrates nearby to the cell membrane. B,C) ADAM10 proteolytic activity is modulated by the interaction with Sfrp1, postulated to interact by dimerization of the respecive cysteine-rich domains (represented in purple). B) Sfrp1-mediated negative modulation of ADAM10 might depend on Sfrp1-NTR interaction with the ADAM10 catalytic domain, thus acting as allosteric competitor. C) Sfrp1-mediated positive modulation of ADAM10 might be dependent of the interaction of Sfrp1 with both ADAM10 and the substrate. This interaction promotes the shedding of the substrate by ADAM10. Derivative work from Wikimedia.

3. Sfrp1 as therapeutic target to counteract neuroinflammation

The results obtained in this study strongly support the idea that interfering with Sfrp1 activity may counteract the detrimental effect of neuroinflammation, suggesting that Sfrp1 could be a promising therapeutic target for the treatment of neurological disorders. Unspecific IgGs or Aβ-specific antibodies have been used in several clinical trials to delay or reduce AD pathology (Lunemann et al., 2015). However, most of them had no or limited success with the exception of that in which patients have been treated with the specific antibody aducanumab against oligomeric Aβ (Sevigny et al., 2016). In this case, patients presented a reduction of Aβ-deposition and a slower progression of dementia. This encouraging study however can be applied only to AD patients. Our approach targeting Sfrp1 to prevent exacerbated neuroinflammation could be applied to a broader range of patients affected by diseases that involve chronic neuroinflammation. This idea is supported by the results of our trial.

In a mouse model of AD, we systemically infused an antibody against SFRP1 to neutralise Sfrp1 activity. After two months, treated animals present a reduced Aβ plaque burden and accumulations of anti-SFRP1 in the cortex resembling Aβ deposits. Two different and compatible effects of anti-SFRP1 may explain this reduction: reduced Aβ deposition and enhanced of Aβ clearance. The first one would result from the neutralisation of Sfrp1 activity as negative modulator of ADAM10 activity. This will enhance ADAM10 processing of APP, thereby preventing Aβ formation. The second one might be triggered by anti-SFRP1 accumulation in the Sfrp1 containing amyloid plaques. “Opsonised-like” plaques would then trigger microglial TREM2-mediated phagocytic activity (Xiang et al., 2016), thereby enhancing plaque clearance. As an additional beneficial effect also TREM2-dependent, microglial clustering around the plaques promoted by anti-SFRP1 antibodies might limit Aβ diffusion (Y. Wang et al., 2016) and impair neuronal dystrophy (Yuan et al., 2016). TREM2 signalling has been also directly related to mediate microglial Aβ uptake by interacting with Aβ-lipoproteins complexes (Yeh et al., 2016). This suggest that Sfrp1 neutralization would limit the progression of the disease reducing Aβ plaque burden and detrimental activation of glial cells, as well as by promoting Aβ clearance and limiting Aβ diffusion by stabilizing TREM2 signalling.

In conclusion, our data support the role of Sfrp1 as modulator of neuroinflammatory responses in the CNS and shows that high Sfrp1 levels may lead to an exacerbated microglial response with detrimental consequences for CNS homeostasis. The relevance of Sfrp1 in microglial priming and senescence indicates that the role of this protein in aging should be studied. Moreover, the possible Sfrp1 substrate-specific regulation of ADAM10 must be confirmed and thereafter verified *in vivo*. Last, but not least, Sfrp1 neutralization might be a promising avenue for the treatment of the neuroinflammatory processes involved in the onset and progression of diverse neurological disorders.

4. Future perspectives: Possible implication of Sfrp1 in age-related diseases

The data exposed in this thesis reveal a novel function of Sfrp1 as a pro-inflammatory modulator of microglial activation. However, the relevance of increased levels of Sfrp1 on other brain cells types has not been assessed. Sfrp1 has been related to the modulation of mesenchymal stem cells (MSC) function in vessel maturation (Dufourcq et al., 2008). As MSC have been implicated in BBB stabilization and the regulation of its permeability (Park et al., 2015), it might be worth studying the possible implication of Sfrp1 in the modulation of BBB permeability. Sfrp1 direct action on neurons should be also considered, since Wnt signalling (Budnik & Salinas, 2011) and ADAM10 (Prox et al., 2013) have been implicated in synapse formation and remodelling, and their inhibition induces synaptic degeneration and cognitive impairment (Marzo et al., 2016; Prox et al., 2013). Sfrp1 impairs synaptic plasticity leading to an aged synaptic phenotype (Gogolla et al., 2009). Both synaptic plasticity impairment (Cerpa et al., 2011) and endothelial-derived BBB disruption (Winkler et al., 2015) are processes widely related to the onset and progression of age- and neurodegenerative-related cognitive decline.

One of the principal theories of aging states the importance of inflammation in aging events. The so-called “Inflammaging” seems to involve the upregulation of pro-inflammatory mediators in the circulation. Inflammaging is described as a systemic, low-graded, chronic inflammation in absence of infection (Franceschi et al., 2000) that is present in aging individual and it is thought to facilitate neurodegenerative disorders (Cribbs et al., 2012). Nonetheless, the specific aetiology of inflammaging remains unknown, but cellular senescence has been described among its most likely sources (Franceschi & Campisi, 2014).

Senescence is a response to damage and stress that supresses the proliferation of tumorigenic cells (Campisi & d'Adda di Fagagna, 2007). Persistent senescent cells are thought to drive aging and age-associated pathologies through an altered protein secretion profile, which is called the senescence-associated secretory phenotype (SASP) (Coppe et al., 2010). SASP factors include several pro-inflammatory cytokines and chemokines, growth factors and regulators. Likewise, Sfrp1 has been described as a SASP, necessary and sufficient to induce senescence phenotypes through inhibition of Wnt signalling (Elzi et al., 2012). This fit well with the role of Sfrps as tumour suppressors genes (Surana et al., 2014) and is supported by the strong evidence of the inverse association between cancer and neurodegenerative diseases (Driver, 2014). Sfrp1 increased expression mediates the appearance of glaucoma (W. H. Wang et al., 2008) inducing the senescence of trabecular meshwork cells that regulates the intraocular pressure (Morgan et al., 2015). Glaucoma is considered an aging-associated disease, and the strong association with AD have suggested glaucoma to represent a manifestation of AD (Lai et al., 2017). In this direction, the appearance of senescent dystrophic microglia has been related to represent a major contributor to the onset of sporadic AD (Streit et al., 2009). All these data consistently supports the implication of Sfrp1 in the induction inflammaging.

In this work, we have demonstrated that increased levels of Sfrp1 locally in the CNS induce microglial activation but circulating Sfrp1 could also promote microglial priming. Growing evidence associate peripheral diseases characterized by elevated systemic inflammation, such as obesity, rheumatoid arthritis or diabetes type 2, with a higher risk of developing cognitive decline (Marioni et al., 2010). It is possible that low-graded chronic systemic inflammation triggers microglial priming, but both acute and chronic systemic inflammation have been shown to increase the rate of cognitive decline in patients with AD (Holmes et al., 2009). Related to that, unpublished data from our laboratory indicate that Sfrp1 levels are increased in serum samples from elderly people. In the same direction, Sfrp1 is one of the most significantly up-regulated (almost 200-fold change) genes in monocytes of aged rats, together with a large number of recognised markers of cytotoxicity, inflammation and migration (Martinez et al., 2015). The increase of both, brain- and/or circulating-Sfrp1 levels, could represent an

inflammatory stimulus that triggers microglial priming to exacerbate microglial response, once known the CNS pro-inflammatory effect of Sfrp1 described in this work.

In addition and related to the increased levels of circulating Sfrp1 during aging, it has been demonstrated the importance of circulating factors in the induction of age-related cognitive impairment (Villeda et al., 2014). This enhances our data indicating Sfrp1 as mediator of neuroinflammatory processes, that directly in the CNS or indirectly systemic as an age-related SASP could promote dysfunction and/or priming of microglia, triggering detrimental chronic inflammatory processes in the CNS promoting neurodegenerative diseases.

CONCLUSIONS

- Sfrp1 is involved in neuroinflammation and its expression is upregulated under pro-inflammatory conditions.

- Sfrp1 upregulation in response to a pro-inflammatory stimulus mostly depends on microglial cells and to a lower extent on astrocytes.

- Overexpression of Sfrp1 is sufficient to induce an inflammatory response.

- Genetic inactivation of *Sfrp1* reduces the response of glial cells to a pro-inflammatory stimulus

- This milder response reduces the symptomatology of EAE and delays AD progression.

- Sfrp1 seems to modulate ADAM10-mediated processing of different molecules involved in the onset of microglial activation towards a pro-inflammatory state.

- Sfrp1-mediated modulation of ADAM10 proteolytic activity seems to be substrate-dependent.

- Sfrp1 represents a potential therapeutic target to ameliorate exacerbated neuroinflammatory processes characteristic of neurodegenerative diseases.

CONCLUSIONES

- La proteína Sfrp1 está implicada en procesos neuroinflamatorios y su expresión está aumentada bajo condiciones pro-inflamatorias.

- El incremento de la expresión de Sfrp1, en respuesta a un estímulo pro-inflamatorio, depende en su mayoría de células microgliales y en menor medida de astrocitos.

- La sobreexpresión de Sfrp1 es suficiente para inducir una respuesta inflamatoria.

- La inactivación genética de *Sfrp1* disminuye la respuesta glial frente a un estímulo pro-inflamatorio.

- Esta respuesta reducida va asociada a la disminución de la sintomatología asociada a la EAE y retarda la progresión de la EA.

- Sfrp1 parece modular la actividad proteolítica de ADAM10, alterando el procesamiento de diferentes sustratos implicados en la activación de la microglia hacia un estado pro-inflamatorio.

- La modulación de ADAM10 mediada por Sfrp1 parece ser dependiente de sustrato.

- Sfrp1 representa una potencial diana terapéutica para atenuar los exacerbados procesos neuroinflamatorios característicos de enfermedades neurodegenerativas.

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